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in Prostate Cancer Cells

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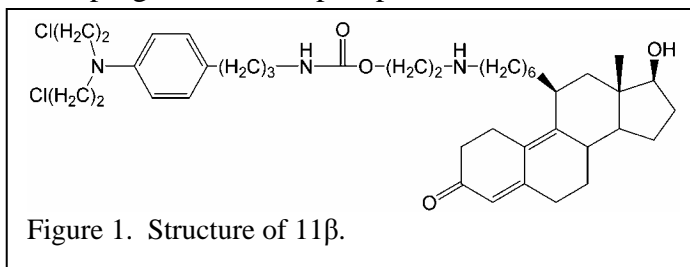
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14. ABSTRACT We have designed and synthesized a novel compound (11 β) that efficiently triggers apoptosis in prostate cancer cells such as LNCaP. This bifunctional compound was designed to form DNA adducts that are camouflaged by the androgen receptor making them less readily repaired in AR+ prostate cancer cells. The aims of our studies are to investigate the mechanisms by which 11 β is able to trigger apoptosis in target cells. One approach we are taking is to prepare structural analogs of 11 β that have increased or decreased abilities to cause apoptosis in LNCaP cells. Methods have been developed that will permit us to determine the fates of 11 β -DNA adducts in treated cells as well as in target and nontarget tissues in xenograft mouse models of human prostate cancer. Another objective is to identify the signaling events that lead from DNA adducts to activation of the apoptotic program. Finally we have obtained encouraging results from animal experiments that indicate that molecules such as 11 β may have clinical potential for the treatment of human tumors.					
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INTRODUCTION

The objective of our research is to develop more effective therapeutics for the treatment of prostate cancers. One novel bifunctional compound (11 β ; Figure 1) that we have compound contains a chemically reactive nitrogen mustard linked to a steroid moiety that binds with high prepared rapidly induces apoptosis in several prostate cancer cell lines *in vitro*. The 11 β affinity to the androgen and progesterone receptor proteins. This compound was designed to create DNA adducts that form tight complexes with these steroid receptors that make the adducts difficult to repair in prostate cancer cells. Preliminary studies of 11 β in cell culture indicated that its effects on prostate cancer cells were different from those of other alkylating agents used in chemotherapy. The apoptotic responses of prostate cancer cells suggested that the 11 β compound might be a useful agent for chemotherapy. The Specific Aims of our research are to understand the fate of 11 β -DNA adducts in treated cells and investigate the mechanisms that lead to apoptosis. We also proposed experiments to assess the antitumor potential of 11 β in animal models of human prostate cancer.



BODY

Task 1: *Chemical synthesis of monochloro and 17 α -methyl analogs of our lead compound, 11 β -(17 α OH-estradien-4(5),9(10)-3-one)-C6NC2-mustard (11 β), and assessment of their toxicity in prostate cancer cells in vitro.*

The work described in Task 1 has been completed as described in the FY 2004 and FY 2005 Progress Reports. In summary, the two compounds that we proposed to synthesize – the monochloro and 17 α -methyl analogs of 11 β – were prepared (Figure 2). We then characterized the affinity of these new compounds for the androgen receptor and their toxicity toward LNCaP prostate cancer cells. Our results with the monochloro-11 β indicated that both chlorine atoms are required to induce apoptosis – implying that activation of apoptosis by 11 β cells is dependent upon the formation of intra- or inter-strand crosslinks in

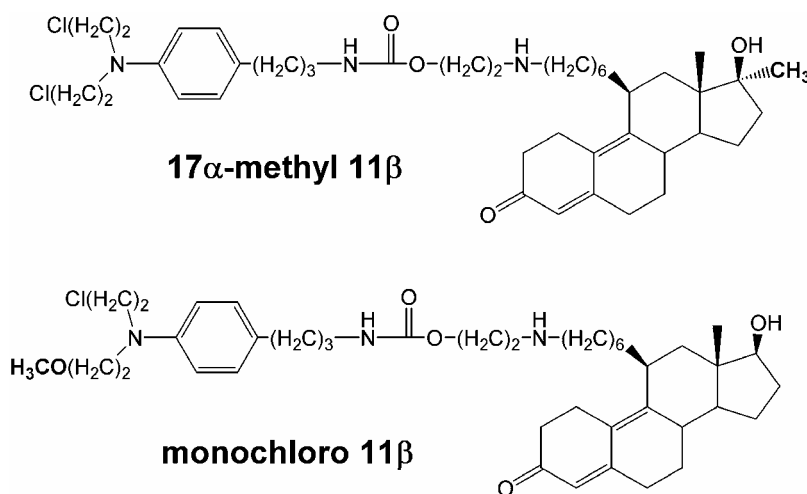


Figure 2. Structures of 11 β analogs prepared in Task 1.

DNA. This result has focused our studies of the fate of 11 β -DNA adducts and directed biochemical studies of the repair of 11 β adducts toward mechanisms responsible for crosslink repair.

We speculated that addition of the 17 α -methyl group to the 11 β molecule would increase its affinity for the AR and result in greater toxicity towards AR-positive prostate cancer cells. Contrary to our expectations the presence of the methyl group has no effect on receptor affinity (both molecules bound to the AR with affinities of approximately 25% that of dihydrotestosterone) or the biological activities of 11 β . Since preparation of the 17- α methyl 11 β compound requires several additional steps we decided not to investigate it further at this time as the original compound can be made more efficiently for *in vivo* studies.

Task 2: *Determine the fate of 11 β -DNA adducts in prostate cancer cells.*

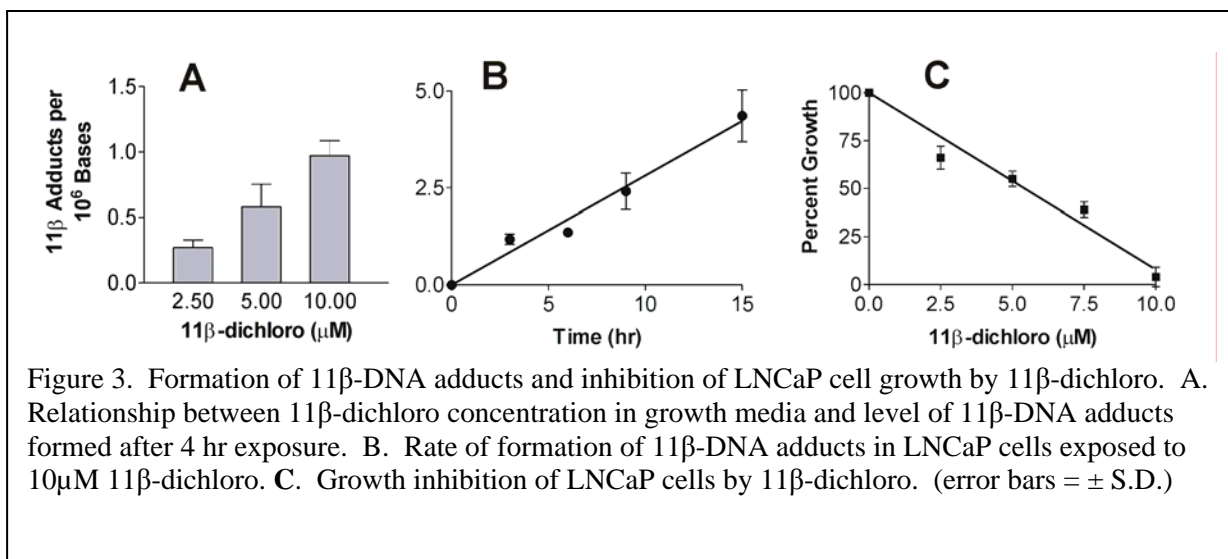
The FY2004 and FY2005 Progress Reports describe our unsuccessful attempts to monitor the kinetics of formation and removal of 11 β -DNA adducts in cells using electrospray ionization mass spectrometry (EIMS). The EIMS technique did not provide the level of sensitivity require for our analyses. Subsequently, we developed an extremely sensitive method for analysis of 11 β -DNA adducts based on the technique of Accelerator Mass Spectrometry (AMS). The AMS technique is up to 7 orders of magnitude more sensitive than conventional liquid scintillation counting. This level of sensitivity has permitted detection of as little as a few hundred DNA 11 β adducts per cell after treatment with [^{14}C]11 β . Thus, we have been able to quantify DNA adduct levels in both prostate cancer cells in culture as well as in xenograft tumors in animals after administration of [^{14}C]11 β .

Our investigation of 11 β -DNA adducts first used the EIMS technique to identify the major 11 β -DNA adducts formed in LNCaP cells in culture and in tissues of mice treated with 11 β . We found the N7 atom of guanine to be the major site of covalent modification by 11 β reacted with DNA *in vitro*. We then confirmed that the same adduct was formed in cells exposed in culture and in tissues of 11 β -treated animals. The identification of DNA adducts formed by the 11 β compound in animals is particularly significant since it confirms that our molecule has sufficient stability *in vivo* to penetrate tissues and reach its molecular target intact. A detailed description of these studies is contained in the appended manuscript (Hillier et al. 2006).

AMS was then employed to study the relationship between DNA modification by 11 β and toxicity in cell culture. We treated LNCaP cells *in vitro* with [^{14}C]11 β and examined the relationships between exposure time, dose and level of DNA adducts. DNA isolated from treated cells was analyzed by AMS to determine the amount of covalently bound [^{14}C]-11 β . We first established a dose-response relationship for adduct formation by treating LNCaP cells with 2.5, 5 or 10 μM [^{14}C]-11 β -dichloro for 4 hr. The amount of ^{14}C per μg DNA increased in direct proportion with 11 β concentration in the growth media (Figure 3A). Based on specific activity, the level of 11 β -DNA adducts rose from 0.3 to 1.0 adducts per 10^6 bases over the dose range of 2.5 to 10 μM 11 β .

We also investigated the rate of formation of 11 β -DNA adducts over a 15 hr period in LNCaP cells treated with a single dose of 10 μ M [14 C]-11 β -dichloro. Figure 3B shows that the concentration of 11 β -DNA adducts in these cells increased at a constant rate during the 15 hr period. The slope of the line in Figure 5B implies a rate of adduct formation of 0.25 adducts/ 10^6 bases per hr. This rate of adduct formation correlates well with the observed number of adducts present in LNCaP cells after 4 hr exposure to 10 μ M 11 β -dichloro (as shown in Figure 3A). These results are also consistent with the stability of the 11 β -dichloro compound in cell culture media since rapid destruction of the compound by hydrolysis or metabolism would be expected to decrease the rate of 11 β adduct formation.

Figure 3C shows the dose-response relationship for growth inhibition of LNCaP cells by 11 β -dichloro. The ED₅₀ for growth inhibition calculated from these data is 5.3 μ M.



We next investigated tumor concentrations of the 11 β adducts in an animal xenograft model. Mice bearing xenograft LNCaP prostate tumors were administered a single dose of 50 mg/kg [14 C]-11 β -dichloro (specific activity = 0.5 mCi/mmol). After 4 hrs, samples of tumor and liver tissues were obtained and DNA was isolated and subjected to 14 C AMS analysis. The results of these analyses are presented in Table 1. Based on the number of amol 14 C/ μ g DNA, tumor tissue had approximately 12% of the concentration of 11 β -DNA adducts as were found in the liver (Table 1). Since AMS analysis cannot reveal the identity of the radiolabeled species, it will require further investigation to confirm that the results of our analyses represent the presence of the 11 β -guanine adduct in tumor tissues. Nonetheless, these data ascertain the ability of 11 β -dichloro to react with one of its intended molecular targets (DNA) in tumor tissue.

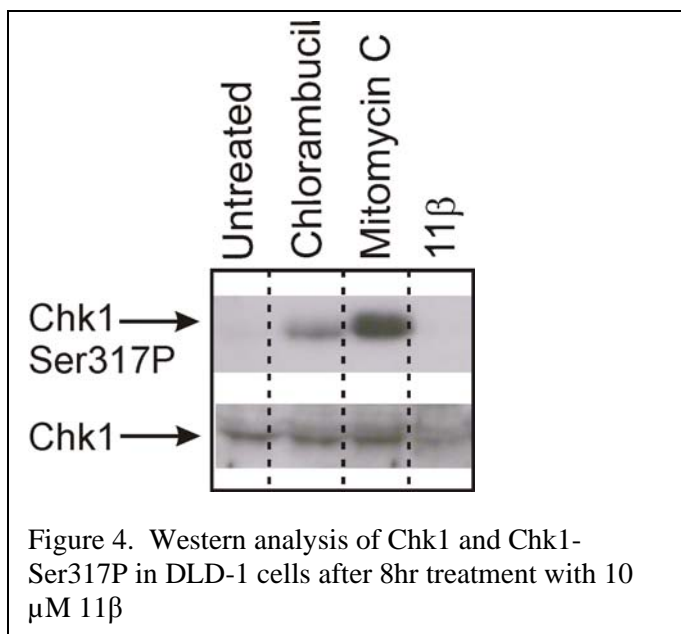
We found that the levels of 11 β -DNA adducts formed in LNCaP xenograft tumors after a single administration of 11 β -dichloro was in the range of the adduct levels associated with concentrations of that inhibited the growth of LNCaP cells in culture. The ED₅₀ for growth inhibition of LNCaP cells in culture by 11 β -dichloro is 5.2 μ M. Approximately

0.25 adducts per million DNA bases were found in LNCaP cells after a 4 hr exposure to 2.5 μ M 11 β -dichloro in culture. A single dose of 50 mg/kg 11 β -dichloro resulted in a similar level of DNA damage in LNCaP xenografts (average = 0.2 adducts/10⁶ bases). These results suggest that the adduct levels reached in tumor tissue are within the range in which toxic effects are observed in cell culture. This is an important finding that suggests we may be able to correlate biochemical responses to adducts that we observe *in vitro* with tumor responses *in vivo*. The results of these studies are discussed in greater detail in the appended manuscript (Hillier et al 2006).

Task 3: *Determine which apoptotic pathways are activated in prostate cancer cells responding to 11 β .*

One aim of our experiments was to uncover the reasons for the ability of 11 β to overcome the resistance of LNCaP and other prostate cancer cells to other alkylating drugs. We began to investigate the pathways and mechanisms that lead to apoptosis by identification of the initiator and effector caspases that are activated by 11 β . Initial experiments found that the pan-caspase inhibitor ZVD.fmk prevented cleavage of PARP confirming that caspase activation was, in fact, triggered in LNCaP cells by 11 β .

As discussed above (Task 1) the results of our structure-activity studies indicated that intra- or interstrand DNA adducts were required for activation of apoptosis. Based on these findings we focused on attention on the consequences of 11 β -DNA adducts on DNA repair and cell cycle check points which are key to cell survival or cell death. Our previous investigations (see FY2005 Progress Report) found evidence that 11 β treatment led to activation of the p53 pathway through phosphorylation of ser15 on p53. We also observed Chk-1 and Chk-2 phosphorylation, but only after 16hr when apoptosis was well underway. During the past year we have examined activation of these checkpoints by 11 β in greater detail. Using DLD-1 cells as a model, we found that Chk-1 was efficiently activated by both chlorambucil and mitomycin C – both of which form crosslinks in DNA that are similar to those formed by 11 β . Neither of these compounds, however, induced apoptosis in LNCaP or DLD-1 cells. When DLD-1s were treated with 11 β we did not observe phosphorylation of Chk-1 or Chk-2 at up to 8hr of treatment (see Figure 4). Inhibition of check point proteins has been shown to sensitize cells to DNA damage. Therefore, we think that it is significant that there is a lack of response of these pathways to damage



produced by 11 β . We are continuing these investigations by testing several hypotheses that could explain these findings including: (1) camouflage of 11 β adducts by their association with the AR or another protein(s) prevents their recognition by DNA repair proteins and subsequent activation of check point kinases; (2) structural features of the 11 β adduct actively prevent formation of DNA repair complexes that are required for check point activation.

Task 4: *Assess the efficacy of 11 β in an animal xenograft model of human prostate cancer.*

As described in our FY 2004 Progress Report, the 11 β compound is very effective in preventing the growth of LNCaP cell in mouse xenografts. It is especially encouraging that the dose of 11 β that was effective in preventing tumor growth did not show significant toxic effects in the animals as evidenced by <10% loss in body weight during an extended treatment regimen. During the last year we have examined the toxic effects of 11 β in greater detail by analysis of serum enzyme markers for hepatotoxicity and erythrocyte levels in blood as a measure of bone marrow toxicity.

Increased levels of serum glutamic pyruvic transaminase (SGPT) and serum glutamic oxaloacetic transaminase (SGOT) were found in animals treated with ≥ 50 mg/kg 11 β -dichloro. After a single administration, we observed no changes in either gamma-glutamyl transferase (GGT) or alkaline phosphatase at doses up to 75 mg/kg 11 β -dichloro (Table 1) while SGPT and SGOT levels were elevated from 35-40 fold at this dose. No significant changes in the serum markers of liver toxicity were found in mice treated with 10 or 30 mg/kg. Furthermore, normal levels of all four serum enzymes were found in animals that had received repeated doses during 7 courses of 5 daily doses of 30 mg/kg (Table 1).

Table 1. Serum enzyme levels in mice after single or repeated administration of 11 β -dichloro.

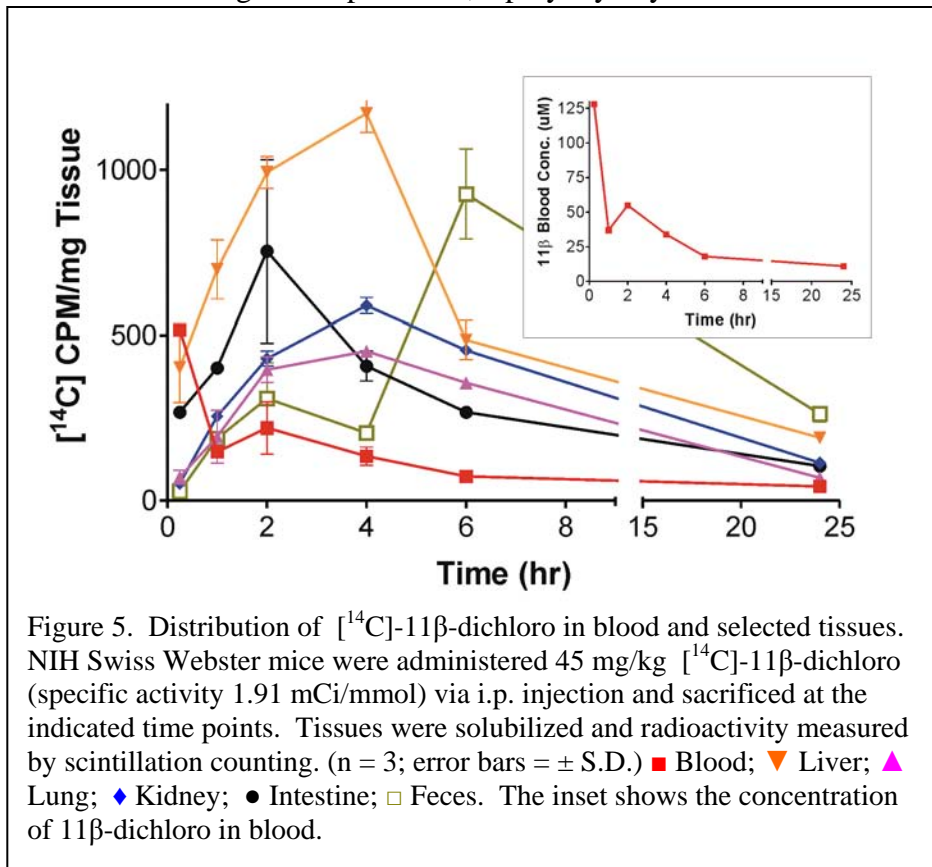
	Single Dose (mg/kg)					Repeated Dosing (mg/kg)	
	10	30	50	75	Vehicle	30	Vehicle
SGPT	50 \pm 24	47 \pm 19	509 \pm 205*	1220 \pm 960*	41 \pm 5	36 \pm 10	48 \pm 10
SGOT	201 \pm 153	105 \pm 35	594 \pm 181*	678 \pm 540*	99 \pm 49	112 \pm 46	119 \pm 33
GGT	<3 \pm 1.7	0 \pm 0	<3 \pm 1.7	<3 \pm 1.7	<3 \pm 1.7	1.8 \pm 0.5	1 \pm 1
Alk Phos	72 \pm 13	77 \pm 10	59 \pm 14	69 \pm 12	90 \pm 12	58 \pm 18	60 \pm 5

NOTE: Enzyme levels were analyzed in whole blood collected by cardiac puncture 24 hr after single doses or from animals after completion of a 7-week protocol during which they received 5 consecutive daily doses per week. (average \pm S.D. (n=3), * P < 0.05; Student's t test).

Analysis of blood reticulocyte levels found that in mice treated for an extended period of time with the 30 mg/kg dose the average level of reticulocytes in treated mice ($6.9 \times 10^6/\text{mm}^3$; SD 2.3) was not statistically different from vehicle-treated mice ($7.5 \times 10^6/\text{mm}^3$; SD 1.3). Taken together these data demonstrate that 11 β -dichloro has acceptable toxicity at the level shown to effectively inhibit tumor growth. These results are reported and discussed in greater detail in the appended manuscript (Hillier et al. 2006).

Our 2005 Progress report described the results of pharmacokinetic studies of 11 β in mice when administered in a vehicle containing Cremophore EL, a polyoxyethylated castor oil.

One reason for this was that Cremophore EL in our current vehicle poses potential problems of clinically significant side effects including acute hypersensitivity reactions and peripheral neurotoxicity. Another reason is that we believe that a more optimal pharmacokinetic profile for 11 β can be obtained by formulating the compound in a manner that slows its uptake by the liver. Our findings



shown in Figure 5 indicate that the compound is rapidly absorbed into the blood following i.p. injection and is subsequently eliminated from the circulation by the liver with excretion primarily via the feces. The blood half-life of 11 β was estimated to be 1.5 hrs. We have sought to extend this half-life to increase the bioavailability of the compound to the tumor tissue. We speculated that greater bioavailability would allow us to decrease the total dose necessary for antitumor activity and reduce side effects.

Reformulation studies revealed that 11 β 's physio-chemical properties were compatible with incorporation into long-circulating PEG-coated liposomes. In preliminary studies we found that the 11 β compound was exceptionally stable when it was incorporated into the hydrophobic core of PEG-coated liposomes. Greater than 90% of the compound

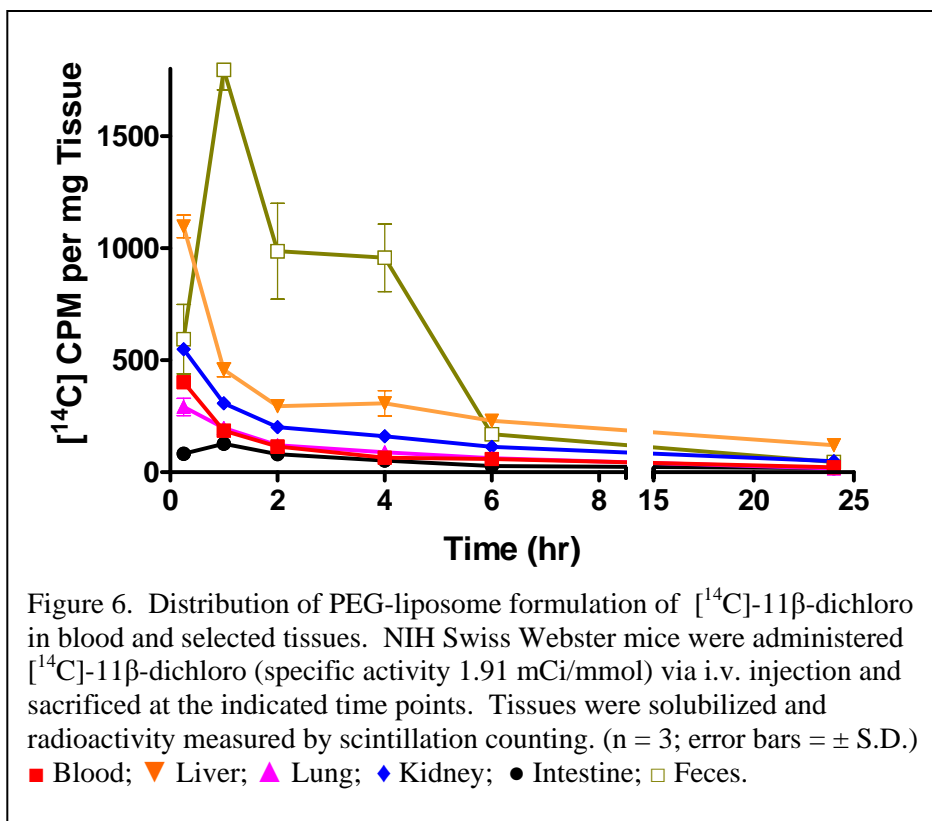
remained intact when 11 β -liposomes were suspended in saline at 37°C for 24 hr. vs a half-life of 2-3 hr when the compound was dissolved in saline and held under the same conditions.

The liposome-based formulations of other hydrophobic anticancer drugs such as doxorubicin and paclitaxel are reported to be effective and have fewer adverse side effects formulations in which these drugs

are administered in Cremophor EL or other vehicles. Our initial studies with liposomal-11 β showed prolonged circulation in the blood over a 10hr period (c.f. FY2005 Progress Report). We continued these investigations with a more thorough analysis of the tissue distribution of 11 β after administration of a PEG-liposome formulation of [¹⁴C]11 β via i.p. injection. Figure 6 shows the distribution of [¹⁴C] 11 β in tissues obtained from mice up to 24hr after administration of the PEG-liposome formulation. These results differ markedly from those of our original study. As shown in Figure 6, there was rapid elimination of the compound from the circulation and excretion in to the gut. In contrast to our initial study we did not see prolonged circulation of the liposomal 11 β in the blood. We think that technical problems in preparing the liposome formulation used in our tissue distribution study may account for the differing results between the two experiments. These experiments were performed in collaboration with the Sasishakran lab here at MIT. We shall continue to investigate the possibility of formulating 11 β into long-circulating PEG-coated liposomes.

We are working with the NCI's RAID program to develop a clinically acceptable formulation for 11 β using a more traditional approach with other vehicles.

Another objective of our *in vivo* studies was to investigate whether the biochemical changes that we observed in cell in culture treated with 11 β were responsible for its antitumor activity in xenografts. LNCaP cells in culture treated with 10 μ M of 11 β rapidly undergo apoptosis (Marquis et al. 2005). Caspase activation is a readily observable maker of apoptosis in tissues. We used immunohistochemistry (IHC) to assess caspase-3 activation in xenograft tumors of animals after treatment with 11 β .



Tumor-bearing animals were administered two courses of 5 daily treatments with 11 β (30 mg/kg) separated by 2 days of recovery. Parallel groups of tumor-bearing animals was treated with chlorambucil (12 mg/kg) or vehicle using the same regimen. Tumors were excised on day 10, fixed and processed for IHC.

Paraffin embedded tissue sections were stained using an antibody against the large fragment of cleaved caspase-3 (Cell Signaling #9664).

Examination of the stained tissue sections revealed a much greater frequency of

activated caspase-3 positive cells in 11 β -treated tumors compared with those obtained from animals treated with chlorambucil (see Figure 7).

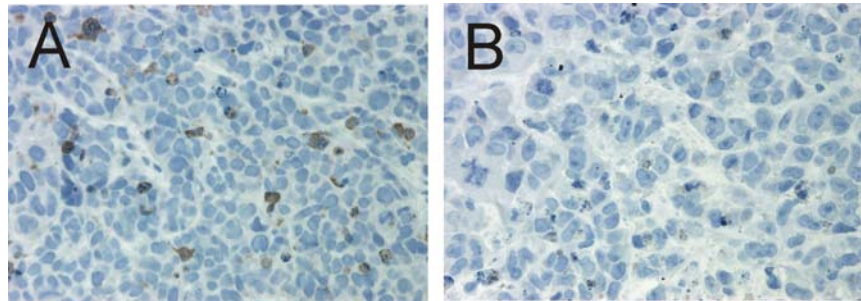


Figure 7. Activated caspase-3 positive cells in xenograft tumors from mice treated with 11 β (panel A) or chlorambucil (panel B).

In summary, our *in vivo* studies have established the efficacy of 11 β in inhibiting the growth of prostate cancer cells in an animal xenograft model. We have also established that effective levels of 11 β that inhibit tumor growth do not result in unacceptable toxicities in the animals. Finally we have important evidence suggesting that biochemical endpoints such as caspase-3 activation and other markers of apoptosis that we can measure in 11 β -treated cells in culture are relevant to antitumor responses. These makers of tumor response should assist us in optimization of treatment regimens.

KEY RESEARCH ACCOMPLISHMENTS

- Evaluation of toxicity of the monochloro-11 β and 17 α -methyl-11 β compounds. The absence of apoptotic responses in LNCaP cells treated with monochloro-11 β indicated bis-DNA adducts are required for apoptosis.
- Optimization of a sensitive method for the kinetic analysis of DNA adducts formed in cells treated *in vitro* with [¹⁴C]-11 β and application of the method to the identification and quantification 11 β -DNA adducts in xenograft tumors. These results have led to experiments that will define the dose relationship between cytotoxic responses *in vitro* and therapeutic responses in animal models. We found that the level of DNA adduct formed by a single dose in tumor tissue *in vivo* was within range in which cytotoxic responses were seen *in vitro*.
- Discovery that Chk-1 and Chk-2 responses are absent in LNCaP cells by 11 β -dichloro while they are activated by other DNA crosslinking agents such as chlorambucil. These results have point to the unique character of DNA lesions formed by 11 β and have led to investigations to identify the molecular basis of the apoptotic responses of LNCaP cells to 11 β -dichloro.
- Definition of the pharmacokinetics of 11 β in mice and preliminary formulation of 11 β into liposomes. Initial experiments have shown promise that the liposomal form of 11 β has increased stability and prolonged circulation in blood. This result has prompted investigation of the pharmacokinetics and efficacy of liposomal 11 β as an antitumor agent in mice.
- Evidence of the activation of apoptosis in xenograft tumor tissue in animals treated with 11 β . This results suggests that our findings in cell culture that 11 β is able to induce apoptosis in prostate cancer cells while traditional alkylating drugs do not have relevance to the antitumor responses we have observed in animal models.

REPORTABLE OUTCOMES

Manuscripts

Shawn M. Hillier, John C. Marquis, Beatriz Zayas, John S. Wishnok, Rosa G. Liberman, Paul L. Skipper, Steven R. Tannenbaum, John M. Essigmann & Robert G. Croy, "DNA Adducts Formed by a Novel Antitumor Agent 11 β -dichloro *in vitro* and *in vivo*". Accepted 2/06 for publication in Molecular Cancer Therapeutics.

Publications

John C. Marquis, Shawn M. Hillier, A. Nicole Dinaut, Denise Andrade, Kaushik Mitra,

John M. Essigmann and Robert G. Croy. 2005 Disruption of Gene Expression and Induction of Apoptosis in Prostate Cancer Cells by a Bis-alkylating DNA Damaging Agent Tethered to an Androgen Receptor Ligand. *Chemistry & Biology* 12; 779-787.

Personnel Supported

Dr. Robert Croy was supported on this grant. He provided supervision and coordination of the research.

Dr. Shawn Hillier has been supported on this grant. He successfully completed his Ph.D. dissertation and was awarded a doctoral degree from MIT in June 2005. He performed pharmacokinetic and antitumor studies of 11 β in animals.

Dr. Kaushik Mitra finished his postdoctoral work in 2005 and is working in drug synthesis at Merck and Co. He was part of the synthetic chemistry team.

Ms. Sreeja Gopal, a graduate student has been supported with funds from this grant. She is investigating the kinetics of 11 β -DNA adducts in cell cultures and animal models.

Dr. John C. Marquis, a postdoctoral student in the lab is investigating the activation of checkpoint proteins and responses of DNA repair pathways to 11 β -DNA adducts.

Funding Applied for based on this work

The research accomplishments from this grant formed the basis of a proposal for continued funding through the DAMD PRCP. The application was successful.

CONCLUSIONS

The Specific Aims of our PCRP Idea Development Award focused on the synthesis of DNA damaging warheads tethered to ligands for the androgen receptor, which is over expressed in most prostate cancers. Out of that work we developed a compound, 11 β , which has become the focus of most of our work over the past two years. As originally proposed, our efforts have focused on understanding the mechanism of action of this novel bifunctional compound. We found that it rapidly induces apoptosis in several prostate cancer cell lines. The compound was designed to create DNA adducts that form tight complexes with steroid receptor proteins resulting in the concealment of damaged sites from repair as well as antagonism the transcriptional activity of the receptor. We have studied the fate of 11 β -DNA adducts in treated cells and investigated the mechanisms leading to apoptosis. We also have done the proposed experiments to assess the antitumor potential of 11 β in animal models of human prostate cancer.

A key research objective was to identify the features of the active 11 β compound responsible for its ability to induce apoptosis in resistant prostate cancer cell lines. We

initially focused our investigations on structure-activity relationships of the reactive N,N-*bis*-(2-chloroethyl)-aniline. Because it has two 2-chloroethyl groups 11 β can form monoadducts, intra- and inter-strand crosslinks. Substitution of one or both chlorine atoms by methoxyl groups produced 11 β analogs that were either unreactive (i.e., both chlorines replaced) or had a single reactive 2-chloroethyl group (i.e. one chlorine replaced) that could only form monoadducts. Investigation of the cytotoxic effects of these analogs revealed that the apoptotic response depends absolutely on the presence of both of the 2-chloroethyl groups in 11 β – implying that apoptosis requires the formation of intra- or inter-strand crosslinks. It is clear, however, that features other than the N,N-*bis*-(2-chloroethyl)-aniline moiety in 11 β are essential, because chlorambucil and melphalan, both of which have bifunctional aniline mustards, fail to induce apoptosis in LNCaP cells. Our studies led to the conclusion that the ability to form bifunctional adducts that interact with a steroid receptor is key to the ability to induce apoptosis.

The formation and fate of 11 β -DNA adducts in prostate cancer cells was another focus of our investigations. To investigate the formation and fate of 11 β DNA adducts in both cell in culture and animal models we developed a highly sensitive method for their analysis that is based on the technique of Accelerator Mass Spectrometry (AMS). A radiolabeled 11 β analog was prepared by modification of its chemical synthesis to allow incorporation of one ^{14}C atom into the linker connecting the steroid and aniline mustard moieties. The sensitivity of the AMS technique – which is up to 7 orders of magnitude greater than conventional liquid scintillation counting – has permitted detection of as little as a few hundred DNA adducts in [^{14}C]11 β -treated cells. In our initial studies with LNCaP cells in vitro we have examined the relationships between both 11 β concentration and exposure time with the level of DNA adducts. The results of these studies will permit us to: (1) examine the rate of repair of DNA adducts in receptor-positive and – negative cells to test the hypothesis that DNA adducts concealed by the androgen receptor are less well repaired; and (2) define the quantitative relationship between adduct level and cytotoxic effects to facilitate optimization of dosing protocols in animal studies.

Another objective of our work has been to understand the mechanism(s) by which the 11 β compound is able to induce apoptosis in cells that are usually apoptosis-resistant. The pan-caspase inhibitor ZVD.fmk was shown to inhibit cleavage of PARP confirming that caspase activation was, in fact, triggered in LNCaP cells by 11 β . We next decided to map the changes in the apoptotic pathways that are downstream of the events resulting from DNA damage. The goal was to investigate the immediate consequences of 11 β -DNA adducts on DNA repair and cell cycle checkpoints – at the beginning of the apoptotic pathways. We examined the activation of checkpoint proteins and found evidence that DNA damage cause by 11 β does not trigger the activation of cell checkpoints. These results are consistent with inhibited DNA repair. We do not know, however, whether this is a result of concealment of adducts by the AR or direct inhibition of signaling pathways required for checkpoint activation. We are investigating these possibilities in additional mechanistic studies.

Our last area of progress has been demonstration of the potent antitumor activity of 11 β in xenograft animal models. We have shown that at tolerable doses the 11 β is a very effective inhibitor of the growth of LNCaP xenograft tumors in mice. We also conducted a pharmacokinetic study of 11 β in mice and have begun to experiments to improve the compound's bioavailability through new formulations. Finally we have begun to connect the unique biochemical changes that we have observed in 11b-treated cells in culture with antitumor responses in animal models. We anticipate that continued pursuit of biochemical mechanisms responsible for the cytotoxic effects of 11 β will allow us to optimize its therapeutic effects.

Prostate tumors are typically refractory to apoptosis induced by DNA damaging agents. By tethering a DNA damaging warhead to a ligand for the androgen receptor, we have designed a molecule that forms DNA adducts that interact with the androgen receptor. These lesions efficiently kill prostate cancer cells in culture by apoptosis, and inhibit tumor growth *in vivo*. These molecules afford a new approach to the treatment of clinically advanced forms of prostate cancer.

REFERENCES

None cited.

APPENDICIES

1. Copy of Marquis et al 2005.
2. Copy of Hillier et al. 2006 Accepted Manuscript to be published in Molecular Cancer Therapeutics.

Disruption of Gene Expression and Induction of Apoptosis in Prostate Cancer Cells by a DNA-Damaging Agent Tethered to an Androgen Receptor Ligand

John C. Marquis, Shawn M. Hillier, A. Nicole Dinaut, Denise Rodrigues, Kaushik Mitra, John M. Essigmann,* and Robert G. Croy*
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Summary

The goal of our work was the design of DNA-damaging agents that disrupt both DNA repair and signaling pathways in prostate tumor cells. A DNA alkylator (*N,N*-bis-2-chloroethyl aniline) was linked to a steroid ligand (17 β -hydroxy-estra- $\Delta^{4(5),9(10)}$ -3-one) to produce a complex molecule (11 β -dichloro) that forms DNA adducts that bind the androgen receptor (AR). We speculated that DNA adducts in an AR-DNA adduct complex would be camouflaged from DNA repair proteins that would otherwise remove the adducts in prostate cancer cells. Furthermore, transcription dependent on the AR would be antagonized by AR redistribution to sites distant from AR-driven promoters. The anticancer potential of 11 β -dichloro was demonstrated against prostate cancer cells in vitro and in vivo. 11 β -dichloro induces a unique pattern of gene disruption, induces apoptosis in apoptosis-resistant cells, and shows promising anticancer activity in animals.

Introduction

Cytotoxic agents that act by covalent modification of DNA were the first modern anticancer chemotherapeutics and remain major components of combination chemotherapy regimens [1, 2]. In combination with drugs that act by other mechanisms, alkylating antitumor drugs have produced impressive and even curative responses in the treatment of some cancers (e.g., cisplatin in testicular cancer) [3]. Frequently, however, tumors are found to have inherent resistance to these compounds or to develop resistance during the course of treatment. The rapid evolution of resistance makes it imperative to develop new agents that can defeat the molecular barriers responsible for clinical failure.

The strategy of combining several agents that can increase the vulnerability of certain cancers to alkylating drugs is one approach to overcoming resistance. Sometimes, however, drug-drug interactions or overlapping side-effect profiles make multidrug regimens problematic.

Another strategy is the design of alkylating compounds with novel mechanisms that disrupt multiple biochemical pathways responsible for tumor growth and survival. We report the design, synthesis, and biological activities of a multifunctional compound based on an original concept that sought to incorporate several

mechanisms of action into a single anticancer agent. Our idea was to produce a compound that could form covalent DNA adducts that have high affinity for a protein essential for the growth and survival of tumor cells. Protein-adduct complexes formed in these cells would not only camouflage the DNA adduct, making it difficult to repair, but also prevent the protein from performing its role in cell growth and/or survival. Figure 1A illustrates these mechanisms showing alternative fates of DNA adducts formed by the reactive “warhead” portion. On the left, exposed DNA adducts that are not bound to proteins through the protein recognition (or “ligand”) domain can be detected and efficiently excised by repair enzymes, limiting potential genotoxic effects. On the right, however, the “ligand domain” (e.g., a steroid) forms a complex with its cognate protein in target cells, camouflaging adducts and interfering with both the repair process and the normal biological function of the protein bound to the adduct. Cells that express proteins that bind to the ligand domain will be more vulnerable to the toxic effects of the synthetic toxins.

The concept that one could use a custom-designed DNA-damaging agent to hijack a nuclear transcription factor required for cancer-cell growth was introduced by the design and synthesis of compounds that produce DNA adducts to which the estrogen receptor (ER) binds with high affinity [4–6]. Breast cancer cells that express high levels of the ER show greater sensitivity toward these compounds. The current paper describes the design, chemical synthesis, and biological activities of compounds that have been designed to form DNA adducts that bind to the androgen receptor (AR), a transcription factor expressed at high levels in many prostate cancers [7]. AR expression is found in primary prostate cancer and is frequently observed in both hormone-sensitive and hormone-refractory forms of the disease [8]. There is currently no effective long-term treatment for the advanced hormone-refractory stage of the disease. Mutations in the AR, along with aberrant expression of coactivator proteins, can allow transcriptional activation in response to antiandrogens and endogenous hormones, leading to cancer progression and therapeutic resistance [9, 10]. The premise of sequestering the AR at sites of DNA adducts was that repair of the genetic damage that is camouflaged in the receptor-adduct complex would be impeded, as would the function (or functions) of the AR responsible for tumor growth.

We report the synthesis and activities of a bifunctional compound composed of *N,N*-bis-(2-chloroethyl)-aniline linked to an 11 β -substituted estra- $\Delta^{4(5),9(10)}$ -3-one. This new compound, 11 β -dichloro (Figure 1B), is capable of producing DNA adducts that interact with the AR. To investigate the mechanism of action of this compound, we prepared an unreactive analog that could not form DNA adducts (11 β -dimethoxy) (Figure 1B). Investigation of the biological responses of LNCaP cells to our 11 β compounds revealed biochemical changes

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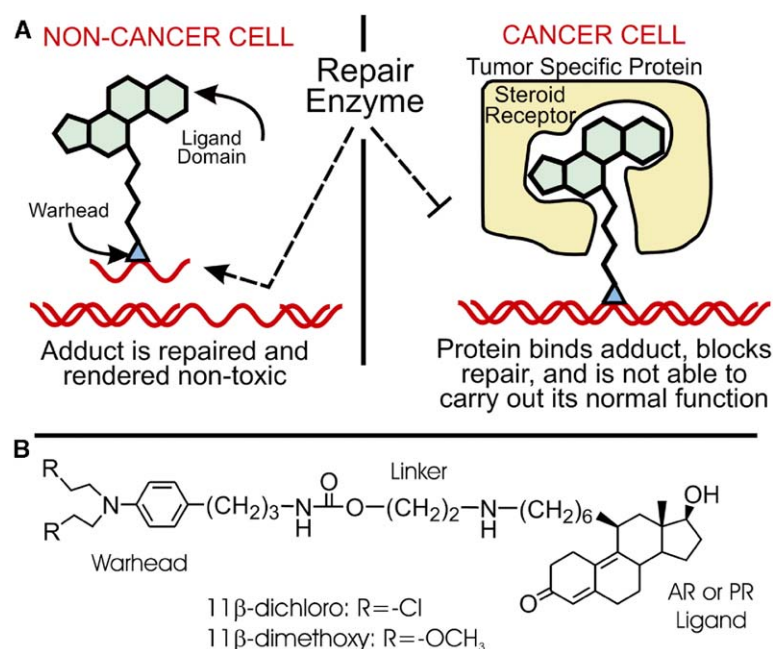


Figure 1. Scheme Illustrating the Mechanism of Selective Toxicity by Compounds that Form DNA Adducts that Attract Proteins in Cancer Cells

(A) 11β-dichloro was designed to have the ability to covalently modify DNA and still retain the ability to bind the protein with which the protein recognition domain was designed to interact. In noncancer cells, which don't express the protein of interest at high levels, adducts are repaired by DNA repair enzymes. In cancer cells, in which the protein of interest is overexpressed, binding of the protein to adducts blocks adduct repair and prevents the protein of interest from carrying out its normal function in the cell. (B) Structure of 11β compounds. 11β-dichloro forms DNA adducts with affinity for the AR and PR. 11β-dimethoxy does not form DNA adducts but does have affinity for the AR and PR.

that are of interest with regard to the antitumor effects we observed in animal models.

Results

Synthesis of Bifunctional 11β-Substituted Estradien-3-One Compounds

The design of the androgen ligand portion of the 11β compounds is based on the reported antiandrogenic activity of molecules containing 11β-substituted 17β-hydroxy-estra-Δ^{4(5),9(11)}-3-one [11]. The general synthetic strategy for linking the 11β-substituted estradien-3-one to *N,N*-bis-(2-chloroethylaminophenyl)-propylamine is shown in Figure 2. Epoxide 1 was prepared by epoxidation of the known compound 3,3-ethylenedioxy-17β-hydroxyestra-5(10),9(11)-diene by a published procedure [12]. The alkyl chain was then introduced at the 11β position by conjugate opening of the allylic epoxide (1) by the Grignard reagent prepared from the alkyl bromide in the presence of copper(I) [13]. Steroid alcohol (2) was converted to the bromide and allowed to react with a protected ethanolamine to give the alcohol (3), which was converted to a carbonate intermediate with *p*-nitrophenyl chloroformate. The carbonate was then

coupled to *N,N*-bis-(2-chloroethylaminophenyl)-propylamine, and the final product, 11β-dichloro (4), was obtained by removal of protecting groups under acidic conditions. Details of some of these procedures have been published [5] and are contained in Supplemental Data. An unreactive analog of 11β-dichloro, in which the two chlorine atoms of the 2-chloroethyl groups were substituted by methoxy groups (11β-dimethoxy) (Figure 1B), was prepared by reflux of 4-(*N,N*-bis-2-chloroethylaminophenyl)-propylamine with NaOCH₃ producing 4-(*N,N*-bis-2-methoxyethylaminophenyl)-propylamine, which was then substituted for its chloro analog in the synthesis as described for 11β-dichloro.

11β-Dichloro Forms DNA Adducts that Interact with the Androgen Receptor and the Progesterone Receptor

Key to the biological activities of our bifunctional molecules is their ability to covalently modify DNA, forming adducts that bind to the androgen receptor (AR). A radiolabeled, self-complementary deoxyoligonucleotide, 5' [³²P]-d(ATTATTGGCCAATAAT)-3', was incubated either with the 11β-dichloro compound or with the unreactive 11β-dimethoxy analog in order to determine the ability

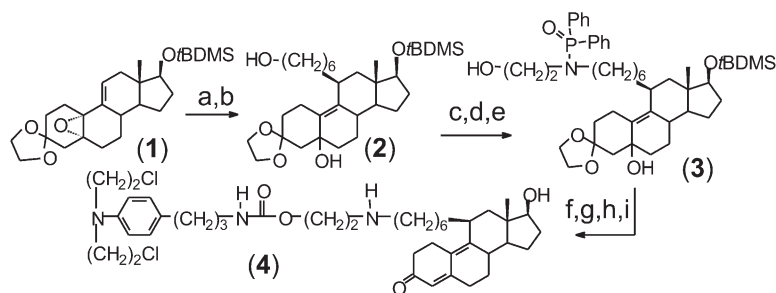


Figure 2. Synthetic Scheme for Synthesis of the 11β-dichloro Compound

(a) *t*BDMSO(CH₂)₆MgBr, CuBr•Me₂S, THF, -20°C; (b) *t*BuNH₄F, THF, room temperature (rt); (c) CH₃SO₂Cl, DIEA, THF, 0°C; (d) LiBr, DMF, 45°C; (e) Ph₂P(O)NH(CH₂)₂OTBDMS, Bu₄NBr, NaH, benzene, 65°C; (f) *t*BuNH₄F, THF, rt; (g) *p*-nitrophenylchloroformate, DIEA, THF, rt; (h) 4-(*N,N*-bis-2-chloroethylaminophenyl)-propylamine, DIEA, THF, 75°C; (i) HCl, THF, rt.

Table 1. Relative Binding Affinities of 11 β -Compounds and 11 β -DNA Adducts for the Androgen Receptor and Progesterone Receptor

Receptor	11 β -Dichloro	11 β -Dimethoxy	11 β -Modified DNA
AR	11.3% (9.7–13.2)	18.1% (15.5–21.1)	0.22% (0.12–0.43)
PR	4.2% (3.5–4.8)	3.6% (3.1–4.3)	0.04% (0.02–0.08)

RBA: relative binding affinity of test compound compared with R1881 (RBA = 100%) for AR or progesterone (RBA = 100%) for PR as determined by competitive binding assay with [3 H]-R1881 (AR) or [3 H]-progesterone (PR); values in parentheses represent the 95% confidence interval for calculated RBAs.

of the compounds to covalently modify DNA. The location of modified bases as well as the extent of modification were determined by piperidine cleavage followed by gel electrophoresis [5]. As expected, the 11 β -dichloro formed covalent products that resulted in fragmentation of the deoxyoligonucleotide at either guanine residue upon incubation with piperidine (data not shown). Approximately 75% of the oligonucleotide treated for 4 hr with 125 μ M 11 β -dichloro was found to be piperidine labile. Under the same conditions, the 11 β -dimethoxy did not produce any piperidine-labile sites.

The relative affinities of the 11 β compounds for the AR and the progesterone receptor (PR) were determined by competitive binding assays. The results of these assays are shown in Table 1. The 11 β -dichloro compound was found to have a relative binding affinity (RBA) of approximately 11% for the AR in whole-cell LNCaP extracts when compared with the synthetic androgen [3 H]-R1881. Because the affinity of R1881 for the LNCaP AR is about twice that of a natural ligand, dihydrotestosterone (DHT) [14], the RBA of 11% implies that the affinity of 11 β -dichloro is approximately 20% that of a natural ligand. Comparing 11 β -dichloro with [3 H]-progesterone, we found that 11 β -dichloro had a much lower affinity for the PR, with an RBA of 4%. Substitution of chlorine atoms by methoxy groups in the 11 β -dimethoxy analog did not influence receptor binding. The RBAs of the 11 β -dimethoxy compound for the AR and PR were similar to those of 11 β -dichloro (Table 1).

We also estimated the RBA for the AR of 11 β -DNA adducts formed by incubation of the self-complementary deoxyoligonucleotide 5'-d(ATTATTGCCAATAAT)-3' with the 11 β -dichloro compound. Addition of 11 β -modified DNAs—but not unmodified DNAs—resulted in decreased amounts of [3 H]-R1881 bound to the AR in cell-free extracts from LNCaP cells. Based on the molar concentration of DNA adducts added, we estimate an RBA of 0.2% for the covalently linked ligand when compared to R1881 (Table 1).

11 β Compounds Induce Growth Arrest and Apoptosis in LNCaP Cells

The biological effects of the 11 β -dichloro and 11 β -dimethoxy compounds were examined in AR-positive LNCaP prostate cancer cells in culture. LNCaP cells exposed to 11 β -dichloro at concentrations >5 μ M underwent rapid morphological changes associated with apoptosis. Figure 3 shows the physical appearance of LNCaP cells left untreated (Figure 3A), after a 6 hr exposure to 20 μ M chlorambucil (Figure 3B), after 6 hr treatment with 10 μ M 11 β -dichloro (Figure 3C), and after 6 hr treatment with 10 μ M 11 β -dimethoxy (Figure 3D). We

selected chlorambucil for comparison because it contains the identical *N,N*-bis-(2-chloroethyl)-aniline moiety as does 11 β -dichloro and would therefore be expected to modify covalently the same atoms in DNA [15]. Despite their chemical similarities, treatment with 11 β -dichloro resulted in striking cytoplasmic contraction and detachment from the culture dish within 6 hr, whereas treatment with chlorambucil did not. Treatment of LNCaP cells with 11 β -dimethoxy produced a transient and less-dramatic change in cell morphology. The cells remained attached to the surface of the culture dish and recovered to their pretreatment morphology by 24 hr.

To determine whether an apoptotic program was activated by 11 β -dichloro, we examined the status of several apoptotic markers in the treated cells. Annexin V staining was used as an early marker of changes in membrane phospholipids that are associated with cells undergoing apoptosis [16]. LNCaP cells treated for various lengths of time with 11 β compounds or chlorambucil were stained with the vital stain 7-amino-actinomycin

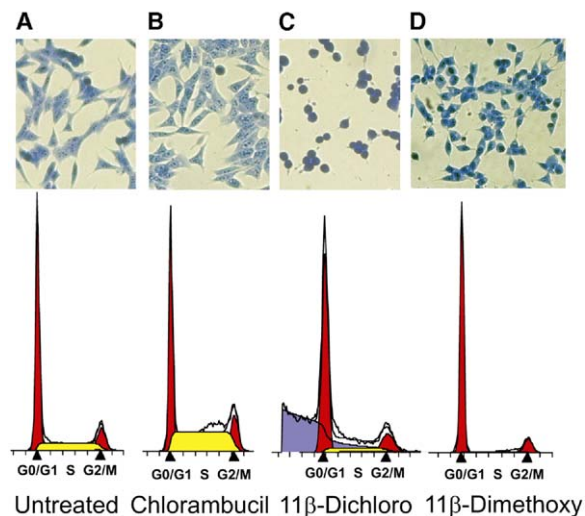


Figure 3. Images of LNCaP Cell Morphology and Cell-Cycle Analysis of LNCaP Cells Treated with 11 β Compounds

Top: LNCaP cells after 6 hr treatment with 11 β compounds (10 μ M) or the anticancer drug chlorambucil (20 μ M). Cells in exponential growth phase were treated for 6 hr, fixed, and stained with Giemsa. (A) Vehicle-treated LNCaP cells. (B) Cells exposed to chlorambucil showed no effect on cellular shape. (C) Cells treated with 11 β -dichloro showed dramatic contraction and detachment. (D) Cells treated with the unreactive 11 β -dimethoxy showed slight contraction, which was reversed by 24 hr (not shown). Bottom: cell-cycle analysis of LNCaP cells treated with indicated compounds for 17 hr.

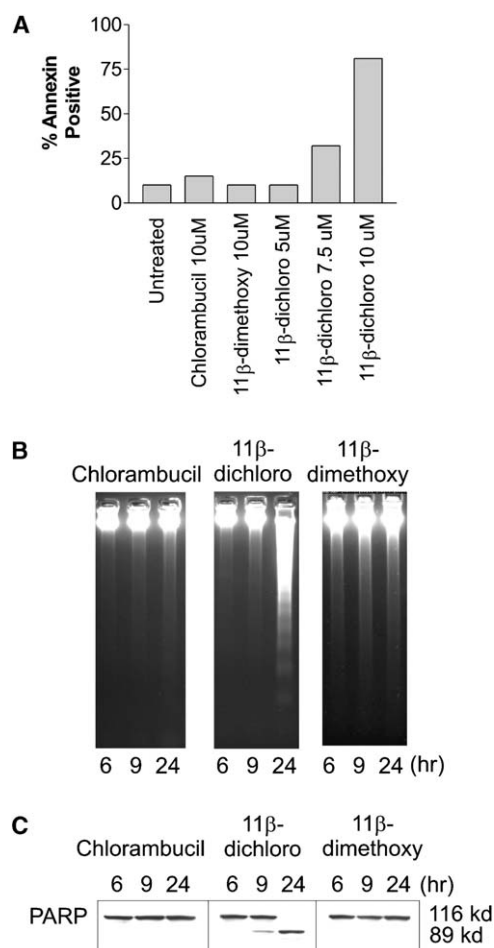


Figure 4. LNCaP Cells Undergo Apoptosis Upon Treatment with 11β-dichloro

(A) Annexin V staining of LNCaP cells after treatment for 15 hr with indicated compounds. Cells treated with $>5 \mu\text{M}$ 11β-dichloro showed evidence of increased Annexin V staining.

(B) Agarose gel electrophoresis of DNA isolated from LNCaP cells after 24 hr exposure to chlorambucil ($20 \mu\text{M}$), 11β-dichloro ($10 \mu\text{M}$), or 11β-dimethoxy ($10 \mu\text{M}$). DNA fragmentation occurs in cells treated with 11β-dichloro.

(C) Western blot analysis of cellular extracts probed with antibodies against full-length and cleaved PARP showed that treatment with 11β-dichloro ($10 \mu\text{M}$) led to cleavage of PARP within 9 hr. No cleavage was seen in cells treated with either chlorambucil ($20 \mu\text{M}$) or 11β-dimethoxy ($10 \mu\text{M}$).

cin D (7-AAD) as well as a phycoerythrin-labeled antibody to Annexin V. Analysis of stained cells by flow cytometry revealed a progression of cells from those that were initially Annexin-positive to a population that stained with both Annexin and 7-AAD, indicating a loss of membrane integrity. These changes were not observed in LNCaP cells treated with either 11β-dimethoxy or chlorambucil (Figure 4A). Analysis of DNA isolated from treated cells revealed the internucleosomal fragmentation and laddering pattern of genomic DNA that is consistent with the activation of an apoptotic endonuclease [17] only in LNCaP cells treated with 11β-dichloro (Figure 4B). DNA that was isolated from cells

treated with 11β-dimethoxy or chlorambucil remained at high molecular weight. Furthermore, cleavage of poly-ADP ribose polymerase (PARP), another apoptotic marker [18], was also seen in LNCaP cells treated with 11β-dichloro but not in cells treated with 11β-dimethoxy or chlorambucil (Figure 4C). These data indicate that 11β-dichloro is clearly effective in overcoming the roadblocks in LNCaP cells that prevent the conventional DNA damaging agent chlorambucil from initiating apoptosis.

Although it did not cause apoptosis, the unreactive 11β-dimethoxy compound did have an effect on cell growth. Analysis of the cell-cycle distribution of LNCaP cells treated with 11β-dimethoxy indicated that the cells arrested in the G1 phase of the cell cycle (Figure 3D). Similar analyses revealed a sub-G1 apoptotic fraction in LNCaP cells treated with 11β-dichloro (Figure 3C), whereas cells treated with chlorambucil were primarily blocked in S phase (Figure 3B).

The results described above led us to investigate the expression of the G1 cell-cycle checkpoint proteins, specifically the cyclin-dependent kinase (CDK) inhibitors p21 and p27, to determine their involvement in the growth arrest and apoptotic responses of LNCaP cells treated with the 11β compounds. Figure 5A shows the levels of these two CDK inhibitors in LNCaP cells exposed for up to 15 hr with 11β-dichloro, 11β-dimethoxy, or chlorambucil. The levels of p21 were affected by all three compounds. Amounts of p21 initially decreased in cells treated with either of the 11β compounds. This initial reduction was followed by either recovery of p21 protein to a basal level in cells treated with 11β-dimethoxy (6–9 hr) or an increase by several fold in cells treated by 11β-dichloro (15 hr). In response to chlorambucil, the amount of p21 showed a constant increase throughout the 15 hr period. Thus, p21 levels were increased at 15 hr in cells treated with either compound that was capable of forming DNA adducts but not in those treated with the unreactive 11β-dimethoxy. The expression level of p27 was unchanged by treatment with chlorambucil. Both of the 11β compounds, however, increased the level of p27 expression.

Given that our compound design was based on the tethering of two distinct chemical entities—a ligand binding portion similar in structure to the PR and AR antagonist mifepristone (RU486) and a DNA-damaging moiety similar to the nitrogen mustard chlorambucil—we were interested in learning if the rapid changes we observed in the levels of the two CDK inhibitors could be reproduced by treatment with RU486 alone or in combination with DNA damage caused by chlorambucil. Figure 5B clearly illustrates the need for the two entities to be linked. As previously determined, chlorambucil increased expression levels of p21. However, treatment with chlorambucil alone, RU486 alone, or a combination of the two did not affect the levels of p27 in cells during exposures up to 15 hr. In addition, cell-cycle analysis of LNCaP cells treated with the combination of RU486 and chlorambucil did not reveal any evidence of nuclear fragmentation (cells with a sub-G1 DNA content) suggesting that—similar to treatment with chlorambucil alone—this combination was not capable of inducing apoptosis (data not shown).

The levels of p27 can be regulated by the protein's

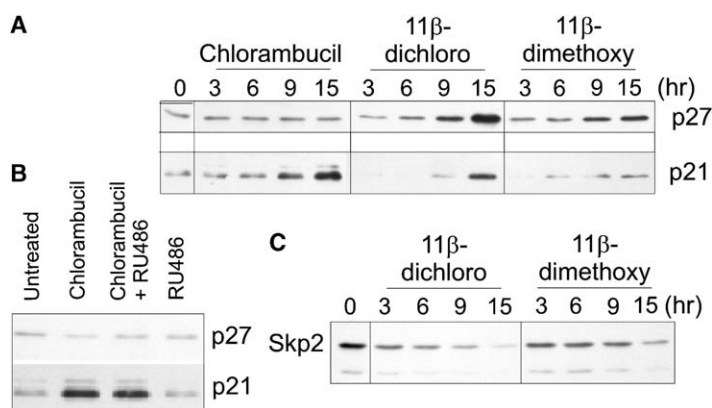


Figure 5. Immunoblot Analysis of Cell-Cycle Checkpoint Proteins in LNCaP Cells

(A) Levels of p27^{CIP1} and p27^{KIP1} in extracts from LNCaP cells that were treated with chlorambucil (20 μ M), 11 β -dichloro (10 μ M), or 11 β -dimethoxy (10 μ M) for up to 15 hr. (B) Levels of p27^{CIP1} and p27^{KIP1} in extracts of LNCaP cells treated for 15 hr with chlorambucil (10 μ M), RU486 (10 μ M), or both (each at 10 μ M). (C) Levels of Skp2 in extracts of LNCaP cells treated under the same conditions as in (A).

ubiquitin-dependent degradation through the ubiquitin E3 ligase SCF^{SKP2} [19, 20]. Therefore, it was of interest to probe the effects of 11 β -dichloro and its dimethoxy analog on the expression levels of Skp2, the F box protein component of the SCF ubiquitin ligase complex. Figure 5C shows the effects of both compounds on Skp2 levels in LNCaP cells treated for up to 15 hr. Treatment with either of the 11 β compounds resulted in decreased expression of Skp2. The decreases in Skp2 expression correlate well with the relative levels of increase in the expression of p27 in LNCaP cells treated with the 11 β compounds. The reciprocal pattern of expression of these proteins shown in Figures 5A and 5C is consistent with the view that Skp2 is directly involved in the increased expression of p27 in LNCaP cells treated with these compounds.

11 β -Dichloro Inhibits Growth of LNCaP Cells in a Mouse Xenograft Model of Human Cancer

The sensitivity of LNCaP cells in culture to 11 β -dichloro led us to investigate its antitumor activity against LNCaP cells growing as xenografts in nude mice. Preliminary toxicology studies in nontumor-bearing mice found that a consecutive 5-day schedule of 30 mg/kg was well tolerated by the animals as evidenced by minimal weight loss and lack of elevated liver transaminase levels (data not shown). Tumor-bearing animals were treated on a schedule of seven consecutive weekly 5-day cycles with a daily dose of 30 mg/kg administered by intraperitoneal injection. Control animals received vehicle only. As shown in Figure 6, this regimen resulted in a 90% inhibition of tumor growth when assessed on the final day of the study (mean tumor volume [treated] versus mean tumor volume [control] on day 45; $p < 0.0001$). During the 45-day period, the treatment group experienced a mean weight loss of 9.7% compared with the vehicle-treated group. These data demonstrate that at tolerable doses, 11 β -dichloro inhibits the growth of LNCaP prostate cancer cells in vivo. The tumoristatic effect that we observed in this model may be due to the relatively small initial size of the tumors (5 mm diameter) at the initiation of treatment with 11 β -dichloro. We are investigating the responses of larger, more established tumors to determine whether the cytotoxic responses observed in vitro occur in xenografts resulting in the reduction in tumor size.

Discussion

In this report, we examined the effects of a new, rationally designed, multifunctional compound on the growth of LNCaP prostate cancer cells in culture and in a xenograft animal model. We programmed our compound to produce DNA adducts that would be able to form complexes with a protein that was aberrantly expressed in tumor cells. The new compound (11 β -dichloro) comprises a steroid moiety with high affinity for the androgen receptor tethered to a reactive *N,N*-bis-(2-chloroethyl)-aniline. We found that 11 β -dichloro causes increased expression of the cell-cycle checkpoint proteins p27 and p21 along with the rapid induction of apoptosis. Because 11 β contains a DNA alkylator, one could argue that the DNA damage response initiates the observed cellular effects; we did not observe, however, upregulation of p27 or apoptosis with free chlorambucil. Neither did we observe apoptosis with an unreactive 11 β analog that induced p27 and growth arrest at the G1 stage of the cell cycle. The checkpoint and apoptotic responses in LNCaP cells distinguish the

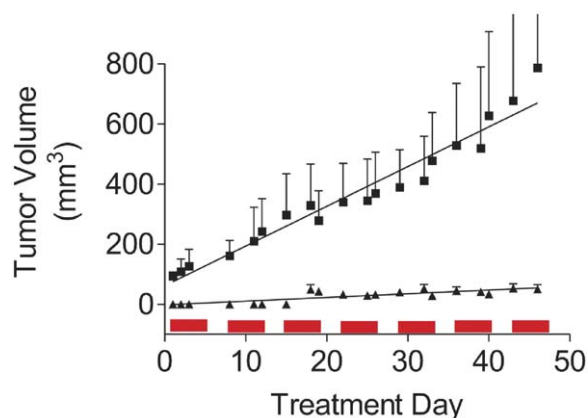


Figure 6. Response of LNCaP Androgen-Dependent Xenografts to 11 β -Dichloro

Bars at bottom each represent five consecutive daily doses of 30 mg/kg. Triangles represent tumor volume in mice treated with 11 β -dichloro; squares represent tumor volume in control mice treated with vehicle only. (Average tumor volume \pm s.d.; $n = 5$)

11 β -dichloro compound from conventional alkylating antitumor drugs.

There are examples in the literature of molecules in which steroid receptor ligands have been combined with chemically reactive genotoxins [21–25]. In most cases, the steroid portion of such molecules is designed to function as a delivery agent and the linker is programmed to hydrolyze and release the active toxin in target cells that express the cognate receptor. In contrast, the linker in our compounds is designed to possess chemical stability as well as to resist hydrolysis by proteases and esterases. Our experiments demonstrate that this manner of linking the two active pharmacophores in 11 β -dichloro resulted in cellular responses distinct from those of the independent compounds.

The results reported here expand on earlier work in which we prepared a group of compounds with the ability to form DNA adducts that can bind the estrogen receptor and selectively kill ER-positive breast cancer cells [4, 5]. Based on the structural similarity of the AR to other steroid receptor proteins [26], we incorporated the molecular features and dimensions that proved successful in the development of our initial ER binding bifunctional compounds into the design of new compounds that have good affinity for the AR. Previous studies established the importance of an alkanyl chain of at least six carbons for binding of modified ligands to the ER. We based our attachment of a six-carbon alkyl chain at the 11 β position of 17-hydroxy-estra $\Delta^{4(5),9(11)}$ -3-one on the reported affinity of the anti-progestin drug RU486 for the AR [27]. The remaining features of the new compound directed at prostate cancer cells were adopted from our previous work [4]. Biophysical characterization of the new molecule found that it had good affinity for the AR of LNCaP cells compared to the natural ligand dihydrotestosterone. The LNCaP cell line has a mutation at residue 877 of the AR that alters its affinity for androgen agonists and antagonists [28, 29]. This mutation has been found in patients with advanced, androgen-independent metastatic prostate cancer [30]. It may be necessary to alter the ligand portion of the 11 β molecule to increase its affinity for other genetic variants of the AR that occur in prostate malignancies.

The 11 β -dichloro compound formed reactive intermediates that produced covalent adducts in DNA. The relative affinity for the AR of these DNA-bound ligands was lower than that of the unreacted 11 β -dichloro compound. This may be because unlike the free compound, the 11 β -modified DNA contains a variety of structures that may have different steric requirements for binding to the AR. Additional investigations are required to determine if a range of affinities for the AR exists for various 11 β -DNA adducts and their relevance to our intended mechanisms of action.

Our studies explored the relationship between the formation of DNA adducts and the ability of 11 β -dichloro to cause cell-cycle arrest and apoptosis. Through chemical substitution, we prepared an unreactive analog of 11 β -dichloro that was incapable of forming DNA adducts. We demonstrated that the unreactive analog (11 β -dimethoxy) was not able to induce apoptosis, although it was able to block LNCaP cells in the G1

phase of the cell cycle, at least temporarily. The results with the control unreactive derivative of 11 β -dichloro shed light on the mechanisms of action independent of DNA damage that contribute to the rapid and dramatic response of LNCaP cells to 11 β -dichloro.

Direct interaction of the 11 β -dimethoxy with the AR in the LNCaP cell line provides one possible explanation for its ability to induce G1 cell-cycle arrest through increased expression of p27, the CDK inhibitor that acts to inhibit G1 cyclin/CDK complexes [31]. The effects of androgens on the proliferation of LNCaP cells are well characterized. Low concentrations of androgens such as DHT stimulate growth, whereas high concentrations are inhibitory [32, 33]. Treatment of growing LNCaP cells with 100 nM DHT has been shown to result in accumulation of p27 and growth arrest within 24 hr [34]. In addition, p27 accumulation during androgen-induced growth arrest has been linked to decreased expression of the Skp2 subunit of the ubiquitin E3 ligase SCF^{Skp2} [20]. The direct binding of Skp2 to the cyclin kinase subunit CKS1 directs the ubiquitination and subsequent proteolysis of p27 [35, 36]. We have uncovered a similar reciprocal relationship between decreased expression of Skp2 and accumulation of p27 in LNCaP cells treated with the 11 β -dichloro compound. In the case of 11 β -dichloro, however, the cells do not arrest in G1 but instead, rapidly undergo apoptosis. This latter property, of course, is of value in an anticancer agent.

During the cell cycle, downregulation of the Skp2 protein is mediated by its APC^{Cdh1}-mediated ubiquitination and destruction [37]. It has been reported that Cdh1 can be activated by some forms of DNA damage [38]. This possibility suggests that 11 β -dichloro may be particularly effective in decreasing Skp2 expression because of its effect on both the AR and DNA-damage response pathways that can control Skp2 expression.

The association of the AR with 11 β -DNA adducts would be expected to antagonize the transcription of genes from AR promoter sequences. Androgen ablation as well as therapy with androgen antagonists that prevent AR-mediated gene expression are effective means to control prostate cancer in its early stages. These strategies fail, however, when mutations activate the AR directly or result in its activation by former antagonists that stimulate cell growth. Sequestration of the AR by 11 β -DNA adducts would be expected to overcome both of these resistance mechanisms.

We also observed the increased expression of another CDK inhibitor, p21, in LNCaP cells treated with either chlorambucil or 11 β -dichloro but not with the unreactive 11 β -dimethoxy analog. p21 plays an essential role in growth arrest after DNA damage [39]. Our findings suggest that p21 expression is modulated by DNA damage produced by the reactive compounds, most likely through activation of the p53 pathway. LNCaP cells express wild-type, functional p53 [40], which is a major regulator of p21 transcription in response to DNA damage [41]. There are several reports indicating that increased expression of p21 protects prostate cancer cells, as well as other cell types, against apoptosis induced by a variety of anticancer agents [39, 42]. These findings suggest significance to the different patterns of p21 induction that we observed in LNCaP cells

treated with chlorambucil or 11 β -dichloro. Although chlorambucil produced a rapid increase in the level of p21, the 11 β -dichloro compound initially decreased levels of p21 in LNCaP cells. Because the initial reduction of p21 levels also occurred with the unreactive 11 β -dimethoxy analog, other features of our compounds, unrelated to DNA damage, are likely involved in this response.

The capacity of p21 to halt cell-cycle progression through inhibition of CDKs, as well as through its interaction with the proliferating cell nuclear antigen (PCNA), which stops DNA synthesis [43], provides an explanation for the accumulation in S phase and the survival of LNCaP cells after treatment with chlorambucil. In contrast, we did not observe an increase in the number of LNCaP cells in S phase during treatment with 11 β -dichloro. Early elimination and delayed increase in the expression of p21 in cells treated with 11 β -dichloro may disable a key checkpoint that normally arrests cells in S phase to allow for the repair of DNA damage, thus sensitizing the cells to apoptosis. Further investigation is required to identify the mechanism (or mechanisms) underlying the initial reduction of p21 in 11 β -treated cells and the role this reduction plays in the cytotoxic effects of our compounds.

The mustard class of anticancer agents includes the first drugs used in modern chemotherapy as well as many that are used daily in the clinic today. The development of new DNA-damaging anticancer drugs, however, has not been the subject of intense research in recent years. Discovery of new anticancer drugs has increasingly focused on targeting pathognomonic changes in cancer cells. The objective of this work was to create multifunctional compounds that combine the modern "targeted" approach with the more traditional methods that are directed against the broad target of DNA replication, upon which tumor cells depend. Selectivity and efficacy of these compounds may not depend on absolute restriction of a single target in a malignant cell but, rather, from the decreased ability of malignant cells, as compared with their normal counterparts, to cope with perturbations in multiple pathways.

Significance

We report the induction of growth inhibition, apoptosis, and antitumor activity by a unique bifunctional conjugate that comprises a DNA alkylator and a steroid ligand for the androgen receptor. The new molecule (11 β -dichloro) was designed to target several biochemical pathways upon which prostate tumor cells depend for growth and resistance to existing anticancer drugs. Unlike previous steroid-alkylator combinations, the conjugate molecule has a chemically stable linkage that confers the ability to form covalent DNA adducts that have affinity for the androgen receptor (AR). We propose that creation of AR-DNA adduct complexes interferes with DNA repair and disrupts AR-mediated transcription and signaling. Evidence of a unique mechanism of action was obtained by investigation of the cytotoxic and biochemical effects of the new conjugate in prostate cancer cells. We observed inhibition of cell growth and rapid in-

duction of apoptosis in LNCaP cells that are resistant to other chemotherapeutics that act through damage to DNA. Cells treated with 11 β -dichloro showed a specific pattern of activation of cell-cycle checkpoint proteins that was not observed with other DNA-damaging agents or with a combination of the steroid and alkylator portions of the molecule. Importantly, 11 β -dichloro blocked the growth of LNCaP xenograft tumors in immunocompromised mice. Our results suggest that stable conjugates that can form adduct-protein complexes in DNA have unique cytotoxic mechanisms that may be useful in treating cancers.

Experimental Procedures

Chemical Synthesis

The bifunctional compounds used in these studies were chemically synthesized in our laboratory. Details of the synthetic steps and characterization of the final compounds by NRM and mass spectrometry are contained in [Supplemental Data](#).

Reaction of 11 β Compounds with DNA

The self-complementary oligonucleotide 5'-d(ATTATTGGCCAAT AAT)-3' was obtained from IDT DNA (Coralville, IA) and was purified by denaturing PAGE. The oligonucleotide was 5' end labeled with [γ -³²P]ATP and allowed to react with test compounds at 37°C for 4 hr. The adducted oligonucleotide was treated with 1 M piperidine for 1 hr at 90°C, and fragments were resolved by denaturing PAGE to determine sites of modification. Reaction products were visualized and quantified by PhosphorImager analysis. The calculated percent cleavage is the proportion of radioactivity in the fragments divided by the total and represents the extent of covalent modification by the test compound.

Relative Affinity of 11 β -Compounds for Steroid Receptors

The relative binding affinities (RBA) of 11 β compounds for the AR and PR were assessed by a competitive binding assay. Whole-cell extracts prepared from LNCaP and T47D cells were used as sources of the AR and PR, respectively. RBAs were determined by addition of increasing amounts of unlabeled test compounds to cell extracts in the presence of radiolabeled ligands ([³H]-R1881, 83.5 Ci/mmol, or [³H]-progesterone 103.0 Ci/mmol; NEN, Boston, MA). The amount of radiolabeled ligand that remained bound to protein after removal of free ligand by adsorption to dextran-charcoal was determined by scintillation counting [4].

Relative Affinity of 11 β -DNA Adducts for the AR and PR

We used an identical competitive binding assay to investigate the ability of 11 β -DNA adducts to bind to the AR and PR. In this case, we used as competitor the covalently modified 16-mer deoxyoligonucleotide prepared as described above. After reaction with 11 β -dichloro, unreacted compound was removed from the modified 16-mer via three consecutive ethanol precipitations. We confirmed the absence of unreacted 11 β -dichloro and estimated the concentration of covalent adducts in the DNA by conducting a parallel experiment with [¹⁴C]-11 β (synthesized in our laboratory; details to be published). Increasing amounts of modified or unreacted DNA were added to cell extracts in the presence of radiolabeled ligands. After incubation, unbound ligand was removed and the amount remaining bound to the receptor determined as described above.

Cell Culture

Cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, MD). The LNCaP cell line was maintained in RPMI 1640 supplemented with 2.5 mg/ml glucose, 10% fetal bovine serum (FBS; Hyclone, Salt Lake City, UT), 2 mM glutamax, 1 mM sodium pyruvate, and 100 mM HEPES. The T47D line was maintained in MEM- α medium containing 10% FBS (Hyclone, Logan, UT), 0.1 mM nonessential amino acids, 100 mM HEPES, 2 μ g/ml bovine insulin, and 1 ng/ml human epidermal growth factor (Invitrogen, Carlsbad, CA). Cells were grown in a humidified 5% CO₂/air atmo-

sphere at 37°C. For studies of cell morphology, LNCaP cells were grown on 13 mm diameter Nunc Thermanox cover slips coated with poly-L-lysine (Invitrogen). At indicated time after treatment, cells were washed twice in PBS, fixed in methanol, air dried, and stained with Giemsa.

Cell-Cycle Analysis

Cells in exponential growth were treated with test compounds dissolved in DMSO. At the indicated times, drug-containing media was removed and detached cells were collected by centrifugation. Attached cells were harvested by trypsinization, pooled with recovered detached cells, and washed once in PBS. Cells were fixed in 70% ethanol and stored at 4°C. For flow cytometry, cells were resuspended in 0.5 ml of a PBS solution containing 0.1% Triton X-100, 0.2 mg/ml DNase-free RNase, and 0.02 mg/ml propidium iodide (Sigma, St. Louis, MO). Cells were analyzed with a Becton Dickinson FACScan flow cytometer with Cell Quest software (MIT Flow Cytometry Core Facility). Data were analyzed with ModFitLT 2.0 software.

Annexin V Staining and Analysis

LNCaP cells in exponential growth were treated with test compounds as described for cell-cycle analysis. At indicated times, cells were trypsinized, washed with PBS, and stained with Annexin V-PE and 7-amino-actinomycin D according to manufacturer's protocols (BD Pharmingen, San Diego, CA). Stained cells were analyzed by flow cytometry.

DNA Isolation and Gel Electrophoresis

Adherent cells were scraped directly into growth media and collected along with any detached cells by centrifugation at 0°C. Cells were lysed in a solution containing 50 mM Tris (pH 8.0), 100 μ M EDTA, 0.5 mg/ml proteinase K, and 0.5% sodium lauryl sulfate. After incubation at 50°C for 3 hr, the lysates were extracted once with phenol chloroform, and nucleic acids were precipitated with ethanol and dissolved in TE (pH 7.5). RNA was digested with DNase-free RNase (Roche Biochemicals, Indianapolis, IN), and the solution was extracted once again with phenol chloroform. DNA was then isolated by ethanol precipitation, and the quantity recovered determined by O.D. 260 nm. Equal amounts of DNA from each sample were loaded onto a 1.5% agarose gel containing 0.1 μ g/ml ethidium bromide and resolved by electrophoresis. DNA was visualized with a UV transilluminator.

Immunoblot Analysis

After exposure to various compounds for indicated times, LNCaP cells were harvested in medium by scraping, washed once in PBS, and suspended in 50 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% NP40, 0.5% Na-deoxycholate, 1 mM Na_3VO_4 , 1 mM NaF, and protease inhibitor cocktail (P8340; Sigma, St. Louis, MO) at 0°C. The cell lysate was centrifuged at 14,000 \times g for 10 min, and supernatants were collected for analysis. Protein concentrations were determined by the Bradford dye binding assay (Bio-Rad Laboratories, Hercules, CA). Lysates were combined with SDS-PAGE sample buffer (0.3 M Tris [pH 6.8], 2% SDS, 1% 2-mercaptoethanol, 10% glycerol), and equal amounts of protein were resolved by SDS-PAGE, followed by transfer to Immobilon-P membranes (Millipore, Bedford, MA). Membranes were blocked with 5% nonfat milk in Tris-buffered saline (0.1% Tween 20, 10 mM Tris [pH 7.4], 150 mM NaCl) and probed with antibody against the protein of interest. Antibody complexes formed with horseradish peroxidase-conjugated secondary antibodies were visualized by chemiluminescence (Supersignal West; Pierce, Rockford, IL). Antibodies were as follows: PARP (06-557; Upstate Biotechnology, Lake Placid, NY), p27 Kip1 (2552; Cell Signaling Technologies, Beverly, MA), p21 (sc-397; Santa Cruz Biotechnology, Santa Cruz, CA), and p45 Skp2 (32-3300; Zymed Laboratories, South San Francisco, CA).

Animal Studies

4- to 6-week-old NIH Swiss nu/nu athymic male mice (25 gm) were obtained from the National Cancer Institute-Frederick Cancer Center (Frederick, MD). Experiments were carried out under guidelines of the MIT Animal Care Committee. Animals were injected subcuta-

neously in the right flank with 5×10^6 LNCaP cells suspended in a solution of 50% PBS/50% Matrigel (Collaborative Research, Bedford, MA). Therapy commenced when a palpable tumor of approximately 4 \times 4 mm formed ($n = 5$ per treatment group). The 11 β -dichloro compound was dissolved in a vehicle composed of cremophor EL, saline, and ethanol (43:30:27). Tumor dimensions were measured with vernier calipers. Tumor volumes were calculated with the formula: $\pi/6 \times \text{larger diameter} \times (\text{smaller diameter})^2$. Statistical analyses were performed with a paired t test. At the end of the study period, animals were euthanized with CO_2 . At the time of sacrifice, blood samples were taken from several animals in each group for a complete blood count, along with serum chemistry and liver function analyses. A complete necropsy was also performed, including histopathology on two animals from each group.

Supplemental Data

Supplemental Data include four figures and can be found with this article online at <http://www.chembiol.com/cgi/content/full/12/7/779/DC1/>.

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DNA Adducts Formed by a Novel Antitumor Agent 11 β -dichloro in vitro and in vivo

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Abbreviations: AR, androgen receptor; i.p., intraperitoneal; s.c., subcutaneous; BEAMS, Biological Engineering Accelerator Mass Spectrometry; HRPC, hormone refractory prostate cancer; ESI-MS, electrospray ionization mass spectrometry; CID, collision-induced dissociation; CH₃CN, acetonitrile; MeOH, methanol; ED₅₀, dose inhibiting growth by 50%; SGPT, serum glutamic pyruvic transaminase; SGOT, serum glutamic oxaloacetic transaminase; GGT, gamma-glutamyl transferase.

Abstract The multifunctional molecule, 11 β -dichloro, consists of a ligand for the androgen receptor (AR) linked to a bifunctional alkylating group permitting it to create DNA adducts that bind the AR. We propose that binding of the AR to 11 β -DNA adducts acts to both shield damaged sites from repair and disrupt the expression of genes essential for growth and survival. We investigated the formation 11 β -DNA adducts in tumor xenograft and non-tumor tissues in mice. Using [^{14}C]-11 β -dichloro we show that the molecule remains intact in blood and is widely distributed in mouse tissues after i.p. injection. Covalent 11 β -guanine adducts identified in DNA that had been allowed to react with 11 β -dichloro *in vitro* were also found in DNA isolated from cells in culture treated with 11 β -dichloro as well as in DNA isolated from liver and tumor tissues of mice treated with the compound. We used accelerator mass spectrometry to determine the levels of [^{14}C]-11 β -DNA adducts in LNCaP cells treated in culture as well as in liver tissue and LNCaP xenograft tumors in treated mice. The level of DNA adducts in tumor tissue was found to be similar to that found in LNCaP cells in culture treated with 2.5 μM 11 β -dichloro. Our results indicate that 11 β -dichloro has sufficient stability to enter the circulation, penetrate tissues and form DNA adducts that are capable of binding the AR in target tissues *in vivo*. These data suggest the involvement of our novel mechanisms in the antitumor effects of 11 β -dichloro.

Introduction

Prostate cancer is the second most commonly diagnosed cancer and the fourth leading cause of cancer death among men in developed countries (1). Most prostate cancers depend on androgens for their growth. Therefore, chemical or surgical castration and/or treatment with hormonal antagonists is often given as adjuvant therapy following the surgical removal of the tumor. Androgen ablation results in the apoptotic death of androgen-sensitive cells producing an initial therapeutic response (2-5). Unfortunately, patients who undergo these adjuvant treatments often develop aggressive and metastatic androgen-independent forms of hormone-refractory prostate cancer (HRPC). New and more effective drugs are needed to treat advanced stages of this disease, as well as to treat more effectively its earlier and less aggressive forms.

The androgen receptor (AR) is expressed throughout the development of prostate cancer and is present in most patients with HRPC (6-9). Clinical evidence suggests that disruption of AR signaling through dysregulation of AR coregulators, AR gene amplification, or mutations in the AR enable it to remain transcriptionally active in the presence of AR antagonists and other therapeutics (10;11). The continued role of the AR in HRPC provides an attractive target for therapeutic development.

We have recently reported the development of a novel cytotoxic agent that was designed to take advantage of the dysregulation of the AR in HRPC (12). 11 β -Dichloro is a bifunctional compound in which an AR ligand is linked to a p-N,N-bis-(2-chloroethyl)aminophenyl moiety that can produce covalent damage to DNA. A consequence of the stable connection between the groups at either end of the 11 β -dichloro molecule is that the AR can bind to covalent adducts that are formed in DNA. It

is hypothesized that these AR-DNA adduct complexes both shield the DNA adduct from repair enzymes as well as prevent the AR from acting (by its normal function) to promote cell growth and survival (Figure 1). This latter mechanism, inactivation of the AR through its physical association with DNA adducts, is different from the mechanism of currently used antagonists.

The 11 β -dichloro compound rapidly induces apoptosis in LNCaP cells in culture at concentrations >5 μ M and is highly effective in preventing the growth of LNCaP tumors as xenografts in mice (90% growth inhibition) (12). The studies described here were designed to examine the tissue distribution of 11 β -dichloro in mice and to determine whether the intact molecule is capable of forming DNA adducts *in vivo* that are required for our proposed mechanisms of action (i.e., adducts capable of interacting with the AR). We have also used the highly sensitive technique of Accelerator Mass Spectrometry (AMS) to investigate the formation of 11 β -DNA adducts in a human prostate tumor growing as subcutaneous (s.c.) xenografts in mice.

Materials and Methods

Chemicals

[¹⁴C]-11 β -Dichloro, (3-{4-[Bis-(2-chloro-ethyl)-amino]-phenyl}-3-[¹⁴C]propyl)-carbamic acid 2-[6-(17-hydroxy-13-methyl-3-oxo-2,3,6,7,8,11,12,13,14,15,16,17-dodecahydro-1H-cyclopenta[a]phenanthren-11-yl)-hexylamino]-ethyl ester, was synthesized with one [¹⁴C] atom in the propyl group connecting the carbamate and the p-N,N-bis-(2-chloroethyl)aminophenyl moieties using procedures described previously

(13). The position of the radiolabel is indicated in Fig 1B. Initial radiochemical purity was >98% and specific activity was 50 mCi/mmol. Unlabeled 11 β -dichloro was used to dilute the radiolabeled material where necessary.

Animals

Four to six week old NIH Swiss Webster mice were purchased from Charles River Laboratories, Wilmington, MA. Female athymic NIH Swiss (*nu/nu*) mice (25 g) were obtained from the National Cancer Institute, Frederick Cancer Center (Frederick, MD). All experiments were performed under the guidelines of the MIT Animal Care Committee.

Dose Formulation

[¹⁴C]-11 β -Dichloro or unlabeled 11 β -dichloro dissolved in a small volume of ethanol was added to cremophor-EL/saline (final proportions, cremophor-EL 37%/ethanol 27%/saline 30%) for administration by i.p. injection.

Bio-distribution Analysis

NIH Swiss Webster mice received a single dose of 45 mg/kg of [¹⁴C]-11 β -dichloro (specific activity 1.91 mCi/mmol) in 50 μ L of vehicle administered via i.p. injection. Animals were sacrificed by carbon dioxide asphyxiation at 0.25, 1, 2, 4, 6, and 24 hours post injection, blood samples obtained by cardiac puncture were collected in heparinized syringes, and the following tissues were excised from each animal: lung, liver, spleen, kidney, intestine and in some instances adipose, heart, and skeletal muscle.

Intestinal contents were also collected. Tissue samples were flash-frozen and stored at -20 °C before processing. Approximately 100 mg of each tissue (10 µL of whole blood) was placed in pre-weighed vials and solubilized in 1 mL of Solvable (Packard Biosciences, Meriden, CT) heated to 65 °C for 3 hours or until the tissue was completely dissolved. The solutions were decolorized by treatment with 200 µL of 30% hydrogen peroxide. After addition of 15 mL Hionic Fluor scintillation fluid (Packard Biosciences, Meriden, CT), radioactivity was determined using a Beckman LS1801 Liquid Scintillation Counter.

Analysis of $^{11}\beta$ in Blood

Two volumes of acetonitrile were added to whole blood and the precipitate was isolated by centrifugation (5 min; 13,000 x g). The amount of $^{11}\beta$ covalently bound to proteins was assessed by determining the [^{14}C] activity in aliquots of the supernatant (organic soluble phase) and precipitate.

The precipitated material was solubilized with 1 mL of Solvable (Packard Biosciences), heated overnight at 70 °C, and decolorized by addition of 200 µL 30% hydrogen peroxide. The fraction of non-covalently bound $^{11}\beta$ -dichloro equals the amount of [^{14}C] in the organic soluble phase divided by the total amount of [^{14}C] in blood.

The amount of intact $^{11}\beta$ -dichloro that was present in blood was determined by HPLC analysis. An aliquot of the organic soluble phase (supernatant) was dried in a Savant SC-110 SpeedVac, dissolved in 100 µL acetonitrile, and injected onto a Beckman ODS 4.6 x 250 mm Ultrasphere column at a flow rate of 1 mL/min with 10% CH_3CN ,

50% MeOH containing 0.1 M ammonium acetate. Compounds were eluted from the column with a 20 min linear gradient that increased the concentration of MeOH to 100%. Compounds were detected by a tandem configuration of UV (Rainin UV-1 UV Detector) and radiochemical (Packard Flow Scintillation Analyzer Model 150TR) detectors. The radiochemical detector was calibrated with a known amount of [^{14}C]-11 β -dichloro that was used to correlate peak area with the amount of [^{14}C]-11 β -dichloro in the sample.

Reaction of 11 β -Dichloro with DNA *in Vitro* and Identification of Covalent Products

Salmon testes DNA was dissolved in 5 mM sodium cacodylate and N,N-dimethylformamide was added to a final concentration of 25%. 11 β -Dichloro was added in DMSO (50 μM) and the solution incubated for 16 hr at 37°C. After phenol:chloroform extraction the DNA was isolated by ethanol precipitation. Covalent products were released by acid hydrolysis in 0.1 N HCl (30 min, 70°C) after which the solution was neutralized with NaOH and adjusted to 20 mM Tris-HCl (pH 7.4) and 10% methanol. The solution was then loaded directly onto a C18 SepPak column (Waters Co.) that was sequentially eluted with 10%, 50% aqueous methanol solutions and finally 100% methanol. After reducing the volume of the 100% methanol fraction, an aliquot was analyzed by HPLC using conditions described above. Compounds in fractions collected from the HPLC were analyzed by electrospray ionization mass spectrometry (ESI-MS) on an Agilent 1100 Series LC/MSD Trap operated in the positive ion mode. Samples were introduced by flow injection (0.2 ml/min) in methanol:10 mM NH_4Ac in H_2O :acetonitrile (50:45:5).

Identification of 11 β -dichloro DNA Adducts *in Vivo*

NIH Swiss Webster mice were treated with 11 β -dichloro (25 mg/kg; i.p.) and sacrificed 4 hours later by asphyxiation with 95% CO₂. Livers were removed surgically, snap frozen on dry ice, and stored at –80 °C. Thawed tissue was minced and homogenized (Dounce) in 3 volumes of cold 0.01 M Tris-HCl (pH 6.9), 0.25 M sucrose, 2 mM calcium chloride (lysis buffer). After filtering through nylon mesh, Triton X-100 was added to a final concentration of 5% and a crude nuclear fraction collected by centrifugation at 1000 x g for 20 min at 4 °C. The pellet was resuspended in 2 volumes of lysis buffer to which sodium dodecyl sulfate and sodium chloride were added to final concentrations of 1% and 1 M, respectively. The viscous solution was extracted twice with chloroform:isoamyl alcohol (24:1) and nucleic acids collected by ethanol precipitation.

RNA was removed by digestion with RNase A. The isolated nucleic acids were dissolved in 2 mL of 0.05 M Tris-HCl (pH 7.5), 0.1 M NaCl and treated with 0.5 mg RNase A (10 min; 37°C). Following extraction with chloroform:isoamyl alcohol (24:1) DNA was isolated by ethanol precipitation. The DNA was then subjected to acid hydrolysis and released products analyzed by HPLC and ESI-MS as described above.

Cell Culture

LNCaP cells were obtained from the American Type Culture Collection (Rockville, MD) and maintained in RPMI 1640 (Gibco) supplemented with 2.5 mg/mL glucose, 1 mM sodium pyruvate, 100 mM Hepes, 2 mM glutamax, and 10% fetal bovine serum in a humidified 5% CO₂/air atmosphere at 37 °C. LNCaP cells were seeded in 6-well plates and allowed to attach to the surface for 24 hr. Cells were then exposed to the

indicated dose of [^{14}C]-11 β -dichloro dissolved in DMSO for the indicated period of time. At the end of the incubation the cells were harvested by trypsinization, pelleted, and washed with phosphate buffered saline (PBS). The DNA from the cellular pellet was isolated according to the procedure described above.

For growth inhibition experiments LNCaP cells were seeded in 6-well dishes at 10^5 cells/well. Forty-eight hr later the test compound was added in DMSO solution. Cells were detached by trypsin/EDTA after 36 hr and the number of cells in control and 11 β -dichloro treated wells determined using a Coulter Counter. The percent growth inhibition is the ratio of cell number in treated and control wells multiplied by 100.

AMS [^{14}C] Analysis of DNA Adducts

AMS analyses were conducted by the Biological Engineering Accelerator Mass Spectrometry (BEAMS) Lab at MIT as described in detail elsewhere (14). DNA concentrations were determined by UV absorption at 260 nm using a Beckman DU-65 UV-Vis Spectrophotometer. Solutions of DNA dissolved in water were applied directly to a CuO matrix used for sample combustion. A standard consisting of a solution of [^{14}C -methyl]bovine serum albumin was used to calibrate the instrument. The amount of [^{14}C] in each sample was calculated from the peak area ratio of the sample to the standard. All samples were analyzed at least twice.

Tumor Implantation and Formation of 11 β -Dichloro DNA Adducts in Xenografts

Tumor xenografts were established on the flank of NIH Swiss *nu/nu* mice by s.c. injection of 2×10^6 LNCaP cells suspended in 0.25 ml of serum-free medium:Matrigel

(Becton Dickinson) (1:1). When the tumors reached about 300 mm³ (5-6 weeks) animals received an i.p. injection of 50 mg/kg of [¹⁴C]-11β-dichloro (specific activity 0.5 mCi/mmol). Four hours later DNA was isolated as described above from liver and tumor tissues of individual mice and subjected to AMS analysis to determine the amount of covalently bound radioactivity.

Analysis of Liver Toxicity in Mice

NIH Swiss *nu/nu* mice injected with either single or repeated dose(s) of 11β-dichloro were sacrificed by carbon dioxide asphyxiation. Blood was collected by cardiac puncture and placed in a Becton Dickinson Microtainer Serum Separator tube for blood chemistry analysis which was performed by IDEXX Laboratories, North Grafton, MA.

Results

Distribution of Radioactivity in Blood and Selected Tissues

[¹⁴C]-11β-Dichloro was rapidly absorbed into the circulation following i.p. injection. The peak concentration of radiolabeled 11β-dichloro in blood of 128 μM was found at 15 min and declined rapidly as shown in Figure 2 (inset) with a half life (t_{1/2}) of 1.3 hr. [¹⁴C]-11β-dichloro was well distributed into the tissues (Figure 2). At 15 min after injection the highest levels of radioactivity were found in the liver, intestine, spleen and lung. High levels of radioactivity were also found in fat (not shown). Tissue concentrations reached maximum levels at 4 hr with the liver and kidney experiencing the highest concentrations. After 4 hr there was rapid accumulation of radioactivity in

feces, which is consistent with biliary excretion as a major route of 11 β -dichloro elimination.

Following organic extraction, less than 10% of radioactivity in blood remained associated with precipitated proteins consistent with a low level of covalent modification of blood proteins. HPLC analysis found that >90% of the organic-soluble radioactivity in blood after 1 hr corresponded to the intact 11 β -dichloro compound (Figure 3). At 4 hr several earlier eluting compounds were observed that are probably 11 β -dichloro metabolites (Figure 3). The majority of the radioactivity (>65%) however, still corresponded to the parent compound.

Identification of 11 β -DNA Adducts *in Vitro*

The presence of the parent 11 β -dichloro compound in blood indicated that the intact molecule could be distributed to tissues and was available to react with cellular DNA forming covalent adducts. A key question concerning the proposed mechanism of action of the new compound is whether the intact molecule is capable of forming DNA adducts *in vivo*. To answer this question we first characterized the DNA adducts formed by the reaction of 11 β -dichloro with DNA *in vitro*. Salmon testes DNA that had been incubated with 11 β -dichloro was subjected to acid hydrolysis and the hydrolyzed material was analyzed by reversed phase HPLC. A single peak was observed that eluted prior to the unreacted 11 β -dichloro compound. Analysis of the eluted material by electrospray ionization mass spectrometry identified an ion with 813.4 m/z (Figure 4 A) corresponding to [M+H]⁺. This ion is consistent with a chemical structure in which one ethylene group at the aniline nitrogen of the 11 β molecule is attached to a guanine base

while the other ethylene has a hydroxyl group substituted for chlorine (Figure 4A, bottom). We did not investigate whether hydrolysis of the chlorine occurred during reaction with DNA or at a subsequent step in our analytical procedure. Further analysis of the 813.4 m/z molecular ion by collision induced dissociation (CID) yielded prominent fragment ions at m/z 662.5 and m/z 372.1 (Figure 4A, inset). The proposed structure of the guanine adduct formed by 11 β -dichloro has a parent ion and CID fragments analogous to those produced by a DNA adduct formed by a structurally related compound E2-7 α in which estradiol is linked to the reactive p-N,N-bis-(2-chloroethyl)aminophenyl group (13). It is likely that the 11 β molecule forms a covalent bond at the N7 atom of guanine, but we did not have sufficient material to allow complete structural characterization.

Analysis of 11 β -DNA Adducts in LNCaP Cells in Culture

Following preliminary characterization of the 11 β -DNA adduct formed *in vitro* we investigated whether the same adduct was formed in LNCaP cells in culture. The 11 β -dichloro compound rapidly induces apoptosis in this AR-expressing human prostate cancer cell line (12). We isolated DNA from LNCaP cells after treatment with 10 μ M 11 β -dichloro for 6 hr and hydrolyzed using the same conditions used for *in vitro* modified DNA. The hydrolyzed products were then analyzed by ESI-MS. Among the hydrolysis products was a prominent ion at m/z 813.5 which was identical to the product identified in DNA that was directly reacted with 11 β -dichloro *in vitro* (Figure 4B). Thus, the 11 β -dichloro molecule remains intact under cell culture conditions and reacts with

guanine bases in cellular DNA. There were no marked differences between the DNA adduct that was identified *in vitro* and the one formed in LNCaP cells in culture.

We next investigated the formation of covalent 11 β -DNA adducts in LNCaP cells by treating them with [^{14}C]-11 β -dichloro. DNA isolated from treated cells was analyzed by AMS to determine the amount of covalently bound [^{14}C]-11 β . The high sensitivity of the AMS technique permitted us to detect and quantify the levels of covalently bound ^{14}C that were present in cellular DNA. We first established a dose-response relationship for adduct formation by treating LNCaP cells with 2.5, 5 or 10 μM [^{14}C]-11 β -dichloro for 4 hr. The amount of ^{14}C per μg DNA increased in direct proportion with 11 β concentration in the growth media (Figure 5A). Based on specific activity, the level of 11 β -DNA adducts rose from 0.3 to 1.0 adducts per 10^6 bases over the dose range of 2.5 to 10 μM 11 β .

We also investigated the rate of formation of 11 β -DNA adducts over a 15 hr period in LNCaP cells treated with a single dose of 10 μM [^{14}C]-11 β -dichloro. Figure 5B shows that the concentration of 11 β -DNA adducts in these cells increased at a constant rate during the 15 hr period. The slope of the line in Figure 5B implies a rate of adduct formation of 0.25 adducts/ 10^6 bases per hr. This rate of adduct formation correlates well with the observed number of adducts present in LNCaP cells after 4 hr exposure to 10 μM 11 β -dichloro (as shown in Figure 5A). These results are also consistent with the stability of the 11 β -dichloro compound in cell culture media since rapid destruction of the compound by hydrolysis or metabolism would be expected to decrease the rate of 11 β adduct formation.

Figure 5C shows the dose-response relationship for growth inhibition of LNCaP cells by 11 β -dichloro. The ED₅₀ for growth inhibition calculated from these data is 5.3 μ M.

Identification of 11 β -DNA Adducts in Liver and Xenograft Tumor Tissue

The effectiveness of 11 β -dichloro in inhibiting the growth of LNCaP prostate tumor cells growing as s.c. xenografts in mice (12) led us to examine the formation of 11 β -DNA adducts in xenograft and normal tissues. First, to establish the identity of adducts that were formed in tissues of mice treated with 11 β -dichloro, we investigated the presence of 11 β -DNA adducts in liver since this tissue is exposed to the highest concentration of 11 β -dichloro (Figure 2). DNA was isolated from liver tissue of mice 4 hr after administration of 11 β -dichloro (45 mg/kg, i.p.), hydrolyzed and analyzed directly by ESI-MS. As in the case of studies in LNCaP cells in culture, we searched for molecular ions of 600 m/z or greater and found one abundant ion of 813.5 m/z (Figure 4C). Further analysis of this ion by CID also produced fragment ions at m/z 662.5 and m/z 372.1. Thus, the DNA adduct formed by direct reaction of 11 β -dichloro with DNA *in vitro* is identical by mass spectrometry to the adduct formed in DNA of cells in culture and in tissues *in vivo*. The fact that the intact 11 β molecule is covalently linked to liver DNA indicates that the molecule has sufficient stability *in vivo* for distribution and penetration into tissues and hence is available to perform its intended biological functions.

Having evidenced formation of DNA adducts by intact 11 β -dichloro *in vivo* we proceeded to investigate tumor concentrations of the 11 β adducts in an animal xenograft

model. Mice bearing xenograft LNCaP prostate tumors were administered a single dose of 50 mg/kg [^{14}C]-11 β -dichloro (specific activity = 0.5 mCi/mmol). After 4 hrs, samples of tumor and liver tissues were obtained and DNA was isolated and subjected to ^{14}C AMS analysis as described above. The results of these analyses are presented in Table 1. Based on the number of amol $^{14}\text{C}/\mu\text{g}$ DNA, tumor tissue had approximately 12% of the concentration of 11 β -DNA adducts as were found in the liver (Table 1). Since AMS analysis cannot reveal the identity of the radiolabeled species, it will require further investigation to confirm that the results of our analyses represent the presence of the 11 β -guanine adduct in tumor tissues. Nonetheless, these data ascertain the ability of 11 β -dichloro to react with one of its intended molecular targets (DNA) in tumor tissue.

Investigation of Hepatotoxic Effects of 11 β -dichloro

An animal xenograft tumor study conducted to investigate the efficacy of 11 β -dichloro against prostate cancer found little overt evidence of toxicity throughout a 45-day 7-course regimen using a dose of 30 mg/kg (12). Because 11 β -dichloro concentrations were highest in the liver and this tissue was also found to experience higher levels of DNA damage than tumor tissue, we investigated whether four serum markers of hepatotoxicity were elevated after single or multiple doses of 11 β -dichloro. In the case of acute exposures, blood was collected from NIH Swiss nu/nu mice 24 hr after they had received a single i.p. dose of 10, 30, 50 or 75 mg/kg 11 β -dichloro. Blood was also collected from NIH Swiss nu/nu mice that had been treated repeatedly with 30 mg/kg 11 β -dichloro on the same regimen used in the xenograft tumor study (12). Serum levels of four enzymes that are frequently elevated in drug toxicity were measured in

treated animals and compared to levels in animals administered vehicle only as well as those of untreated historical controls.

Increased levels of serum glutamic pyruvic transaminase (SGPT) and serum glutamic oxaloacetic transaminase (SGOT) were found in animals treated with ≥ 50 mg/kg 11 β -dichloro. After a single administration, we observed no changes in either gamma-glutamyl transferase (GGT) or alkaline phosphatase at doses up to 75 mg/kg 11 β -dichloro (Table 2) while SGPT and SGOT levels were elevated from 35-40 fold at this dose. No significant changes in the serum markers of liver toxicity were found in mice treated with 10 or 30 mg/kg. Furthermore, normal levels of all four serum enzymes were found in animals that had received repeated doses during 7 courses of 5 daily doses of 30 mg/kg (Table 2).

We also examined blood reticulocytes as a measure of bone marrow toxicity in mice treated for an extended period of time with the 30 mg/kg dose. The average level of reticulocytes in treated mice ($6.9 \times 10^6/\text{mm}^3$; SD 2.3) was not statistically different from vehicle-treated mice ($7.5 \times 10^6/\text{mm}^3$; SD 1.3). Taken together these data demonstrate that 11 β -dichloro has acceptable toxicity at the level shown to effectively inhibit tumor growth.

Discussion

In this study we have identified a covalent DNA adduct formed in cells in culture and tissues of mice treated with a novel bifunctional compound, 11 β -dichloro. A ligand for the androgen receptor linked to a reactive N,N-bis-(2-chloroethyl)aniline mustard comprises the 11 β -dichloro molecule. These chemical features enable 11 β -dichloro to form covalent DNA adducts capable of binding the AR. It is proposed that AR-DNA

adduct complexes interfere with both DNA repair and AR function (see Figure 1). We have shown that 11 β -dichloro rapidly induces apoptosis in LNCaP prostate cancer cells and that the new compound can prevent tumor growth *in vivo* (12). This earlier study found that it is essential to have the DNA damaging and AR ligand moieties in the same molecule in order to observe potent activity against cancer cells.

The results of this study show that, following administration by i.p. injection, the intact 11 β -dichloro molecule forms DNA adducts in cells in culture as well as in mouse tissues after absorption and distribution. Levels of radioactivity were widely distributed in tissues after administration of [¹⁴C]-11 β -dichloro. Chromatographic analysis of radiolabeled compounds extracted from blood revealed that intact 11 β -dichloro was the predominant molecular species present for up to 4 hr. Several unidentified metabolites represented approximately one-third of the radioactivity present in blood at that time.

These findings answer a key question concerning the relevance of our proposed mechanisms of action of 11 β -dichloro to the antitumor effects of this compound *in vivo*. The fact that the compound remains intact and capable of forming DNA adducts in cells and tissues is consistent with the view that its combination of biochemical functions may underlie its ability to prevent tumor growth.

The 11 β -guanine adduct we identified is consistent with the major adduct formed by reaction of bifunctional aniline mustard drugs with DNA (15-18), and is the product of the reaction of one arm of the N,N-bis-(2-chloroethyl) group with a guanine base. Reaction of the second arm of this group with another DNA base can produce intra-strand or inter-strand crosslinks (17-19). Evidence indicates that these bifunctional adducts are primarily responsible for the lethal effects of this class of compounds (20;21). The

efficiency of crosslink formation by nitrogen mustards is not great. Thus, any change in the balance between the removal of 11 β -monoadducts from DNA and their conversion to crosslinks will likely have a significant effect on toxicity. Whether the ability of 11 β -DNA adducts to bind the AR affects this balance requires further investigation.

We found that the levels of 11 β -DNA adducts formed in LNCaP xenograft tumors after a single administration of 11 β -dichloro was in the range of the adduct levels associated with concentrations of that inhibited the growth of LNCaP cells in culture. The ED₅₀ for growth inhibition of LNCaP cells in culture by 11 β -dichloro is 5.2 μ M. Approximately 0.25 adducts per million DNA bases were found in LNCaP cells after a 4 hr exposure to 2.5 μ M 11 β -dichloro in culture. A single dose of 50 mg/kg 11 β -dichloro resulted in a similar level of DNA damage in LNCaP xenografts (average = 0.2 adducts/10⁶ bases).

The measured adduct levels in LNCaP cells within xenograft tumors, however, are likely to vary because of the heterogeneity of the tumor microenvironment. While cells in culture experience uniform exposures to 11 β -dichloro in media, vascularization and rates of perfusion vary within tumors resulting in non-uniform exposures (22;23). Thus, the levels of DNA adducts we identified should be considered an average with some cells likely having greater amounts of DNA damage and cytotoxicity from 11 β -dichloro while others likely experience less.

The levels of 11 β -DNA adducts in liver were 8-fold greater than in tumor tissue. This finding led us to investigate whether single or repeated treatments with 11 β -dichloro resulted in hepatotoxicity. In our examination of four serum enzymes that are diagnostic for liver disease we found significant increases in both SGPT and SGOT occurred at the

50 mg/kg 11 β -dichloro dose. No increases were found in either GGT or alkaline phosphatase. This pattern is consistent with drug induced injury to hepatocytes after the single dose. At a lower repeated dose of 30 mg/kg over a 7 week period with 5 consecutive daily doses, none of the four serum enzymes levels were elevated. The fact that inhibition of tumor growth occurred when animals were treated repeatedly with the 30 mg/kg dose in the absence of significant toxicity is encouraging (12).

We propose that in addition to inhibiting DNA repair, association of the AR with DNA adducts may antagonize AR transcriptional activity. This unique mechanism of disabling AR-mediated gene transcription in HRPC may prove effective against cancers in which over-expression or mutation of the AR, or the changes in AR coregulators underlie escape from androgen blockade (24-26).

Targeting AR function in HRPC is often ineffective because of the variety of mechanisms that enable tumor cells to defeat current antihormonal therapies. Nonetheless, the AR remains an attractive therapeutic target for this disease because of its continued role in the growth and survival of advanced prostate cancers. Elimination of AR function can leave cells vulnerable to other chemotherapeutic agents such as those that act by damaging DNA (7). We propose that 11 β -DNA adducts that capture the AR could provide a novel way of antagonizing its biological functions in HRPC. The combined effects of persistent DNA damage and the unique mechanism of receptor antagonism by 11 β -DNA adducts may result in the disruption of biochemical pathways and provoke prostate cancer cells into apoptosis leading to more effective therapy.

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Table 1. [^{14}C]-AMS analysis of 11 β -DNA adducts in liver tissue and LNCaP tumor xenografts.

Mouse	Liver		LNCaP Xenograft (i.p.)	
	amol $^{14}\text{C}/\mu\text{g}$ DNA	11 β adducts/ 10^6 DNA Bases	amol $^{14}\text{C}/\mu\text{g}$ DNA	11 β adducts/ 10^6 DNA Bases
1	51 \pm 5	1.7 \pm 0.2	4.1 \pm 1.4	0.14 \pm 0.05
2	52 \pm 9	1.7 \pm 0.3	6.3 \pm 0.7	0.21 \pm 0.02
3	45 \pm 5	1.5 \pm 0.2	7.3 \pm 0.7	0.24 \pm 0.02

NOTE: Tissues were obtained from NIH Swiss *nu/nu* mice 4 hr after i.p. administration of 45 mg/kg [^{14}C]-11 β -dichloro (specific activity = 0.5 mCi/mmol). DNA concentration was estimated by UV absorption. The adduct concentration in DNA (adducts per 10^6 bases) was calculated based on the attomoles $^{14}\text{C}/\mu\text{g}$ DNA determined by AMS analysis of the DNA. Results are mean \pm SD (n=3) of DNA samples isolated from mice.

Table 2. Serum enzyme levels in mice after single or repeated administration of 11 β -dichloro.

	Single Dose (mg/kg)					Repeated Dosing (mg/kg)	
	10	30	50	75	Vehicle	30	Vehicle
SGPT	50 \pm 24	47 \pm 19	509 \pm 205*	1220 \pm 960*	41 \pm 5	36 \pm 10	48 \pm 10
SGOT	201 \pm 153	105 \pm 35	594 \pm 181*	678 \pm 540*	99 \pm 49	112 \pm 46	119 \pm 33
GGT	<3 \pm 1.7	0 \pm 0	<3 \pm 1.7	<3 \pm 1.7	<3 \pm 1.7	1.8 \pm 0.5	1 \pm 1
Alk Phos	72 \pm 13	77 \pm 10	59 \pm 14	69 \pm 12	90 \pm 12	58 \pm 18	60 \pm 5

NOTE: Enzyme levels were analyzed in whole blood collected by cardiac puncture 24 hr after single doses or from animals after completion of a 7-week protocol during which they received 5 consecutive daily doses per week. (average \pm S.D. (n=3), * $P < 0.05$; Student's t test).

Figure Legends

Figure 1. A. Scheme illustrating the proposed effects of 11 β -DNA adducts on AR function and DNA repair in target cells. The steroid ligand portion of 11 β adducts could form a complex with the AR that recognition and removal by the DNA repair complex (left). 11 β -DNA adducts compete with the natural ligand for the AR (DHT) and antagonize its transcriptional function leading to diminished gene expression (right). The combination of these mechanisms results in apoptosis and cell death. B. Structure of 11 β -dichloro; * indicates position of ^{14}C atom.

Figure 2. Distribution of [^{14}C]-11 β -dichloro in blood and selected tissues. NIH Swiss Webster mice were administered 45 mg/kg [^{14}C]-11 β -dichloro (specific activity 1.91 mCi/mmol) via i.p. injection and sacrificed at the indicated time points. Tissues were solubilized and radioactivity measured by scintillation counting. (n = 3; error bars = \pm S.D.) ■ Blood; ▼ Liver; ▲ Lung; ◆ Kidney; ● Intestine; □ Feces. The inset shows the concentration of 11 β -dichloro in blood.

Figure 3. Reversed phase HPLC analysis of radiolabeled compounds isolated from blood of mice at 0.25, 1 and 4 hr after administration of [^{14}C]-11 β -dichloro. The peak labeled 11 β -dichloro represents the intact compound. For chromatographic conditions see Experimental Procedures.

Figure 4. ESI-MS analysis of 11 β -dichloro-DNA adducts formed *in vitro* and *in vivo*. (A) Salmon testes DNA reacted with 11 β -dichloro *in vitro*; (B) DNA obtained from LNCaP cells in culture treated with 10 μM 11 β -dichloro for 6 hr; and (C) DNA isolated from liver tissue of NIH Swiss *nu/nu* mice treated with 25 mg/kg (i.p.) 11 β -dichloro. Proposed structures of the most abundant ion (813.5 m/z) and CID fragments (insets) are shown in (A). DNA was subjected to hydrolysis with 0.1 N HCl to release covalent adducts (see Methods).

Figure 5. Formation of 11 β -DNA adducts and inhibition of LNCaP cell growth by 11 β -dichloro. A. Relationship between 11 β -dichloro concentration in growth media and level of 11 β -DNA adducts formed after 4 hr exposure. B. Rate of formation of 11 β -DNA adducts in LNCaP cells exposed to 10 μM 11 β -dichloro. C. Growth inhibition of LNCaP cells by 11 β -dichloro. (error bars = \pm S.D.)

Figure 1

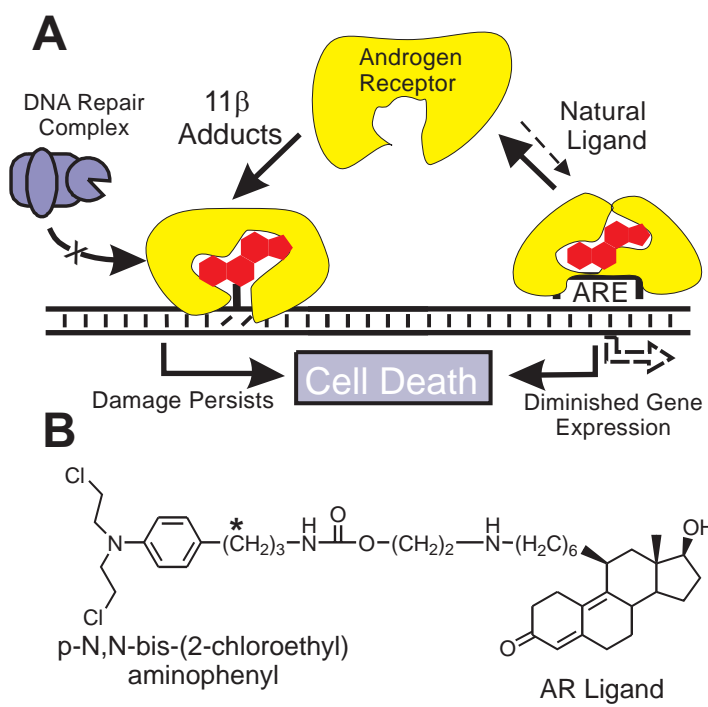


Figure 2

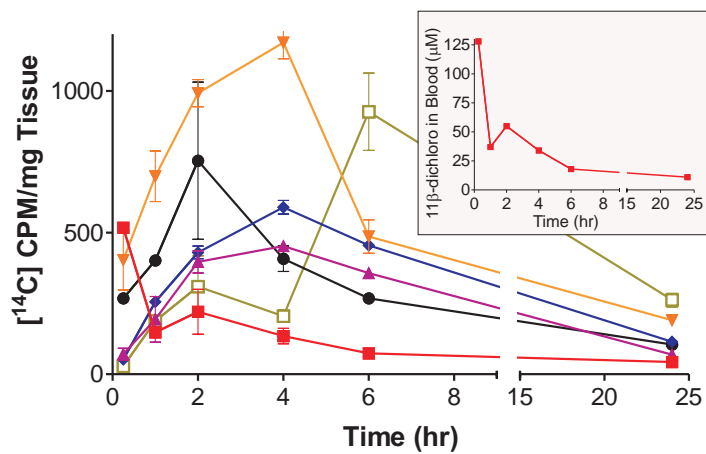


Figure 3

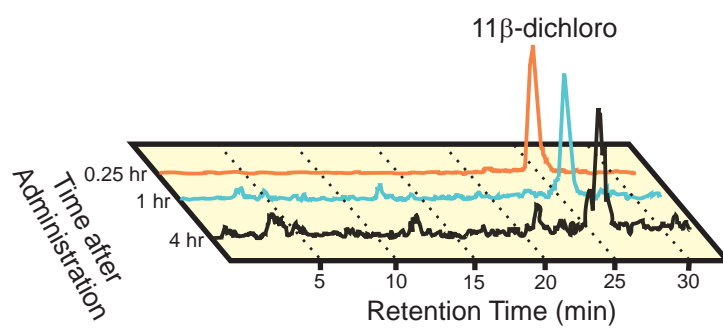


Figure 4

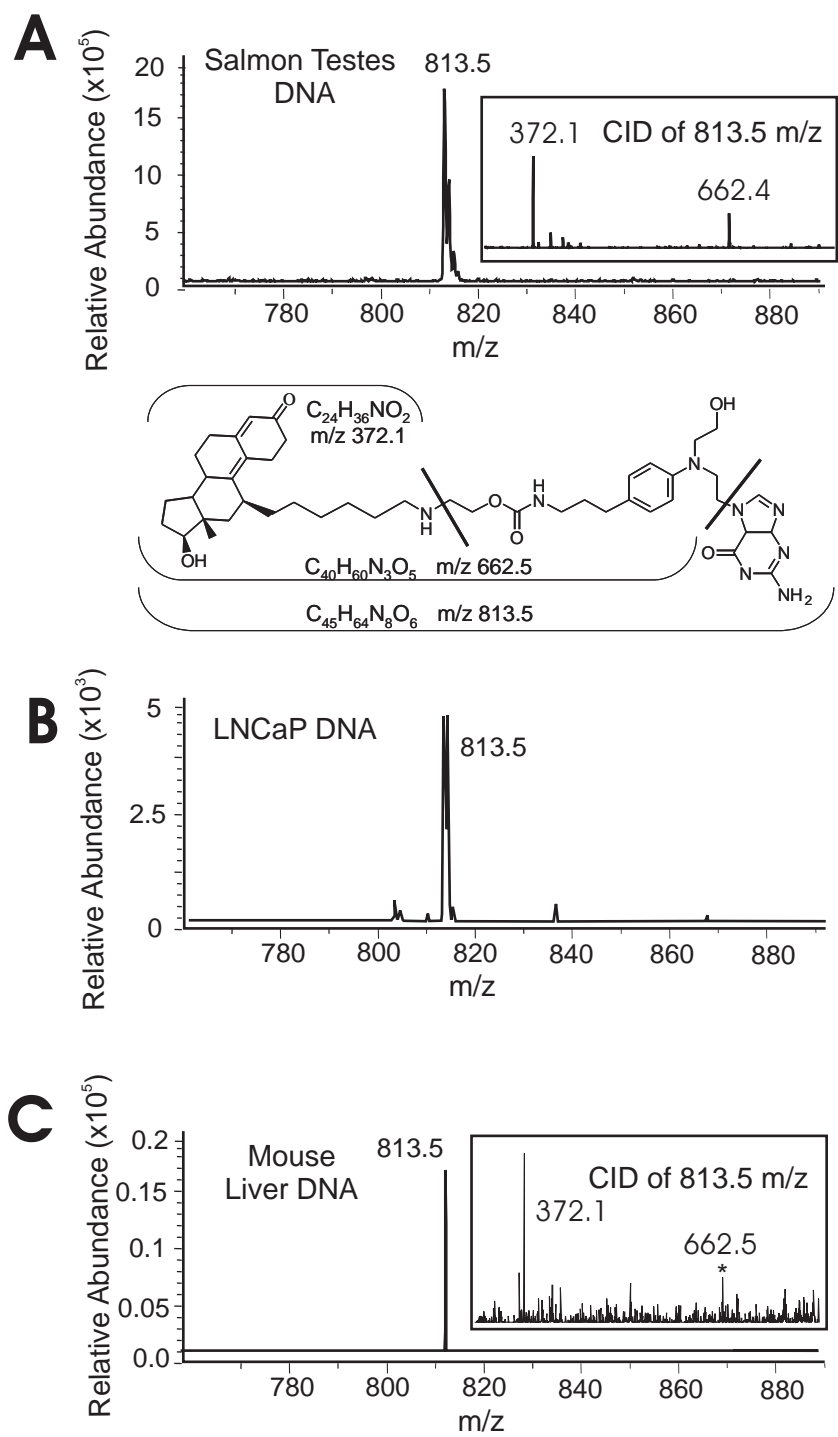


Figure 5

